

Soldiers as experimental animals

The Institute of Medicine in the United States has described a chilling tale of how the military used service people in tests of the effectiveness of poison gases against humans.

THE Cold War is long since over, of course, while now there is a treaty to ban the use of chemical weapons that will, when ratified, decrease the chances that future wars will kill people by poisoning them. But none of that, unfortunately, implies that the horrors of chemical warfare can simply be put out of mind. In recent years, the Kurdish people of Northern Iraq have been forced to learn that lesson at their own government's hands too well for anybody's comfort — especially for that of those of us who live elsewhere. But even if the new chemical treaty puts a stop to what the likes of the government of Iraq has been up to, the consequences of past interest in chemical weapons in places very different from Iraq will cast a long shadow. Those with a ghoulish taste in reading-matter could thus do worse than plough through last month's report from the US Institute of Medicine that catalogues the experiments to study the effects of chemical weapons on human beings carried out in the United States between 1943 and the end of the Second World War (*Veterans at Risk: The Health Effects of Mustard Gas and Lewisite*). Taste apart, the rest of us should also pay attention.

It is a curiously casual tale of bungling indifference to people's welfare. During the First World War, the United States (like its British and French allies) had used mustard gas in retaliation for the first use of this material by the German military. Not much else seems to have happened in the United States until 1941, when the US military set out to relearn what had been forgotten about the effects on people of materials such as mustard gas and its close relatives, lewisite and nitrogen mustard (in which a sulphur atom is replaced by an arsenic and a nitrogen atom respectively). But then, between 1943 and the end of the Second World War, at various times and at different military bases, an estimated total of 60,000 service people were exposed to these agents.

In some cases, drops of the liquid were placed on forearms. In others, troops were asked to manoeuvre on contaminated ground, with or without protection. Yet others were fitted out with protective gear and asked to spend long periods (four hours or so) in gas chambers; some of them were asked to come back the following day. Surprisingly, by present standards of litigiousness, complaints from injured service people (called "veterans") filtered into the US Department of Defense only late in the day, nearly half a century after the event. The Institute of Medicine committee under Dr David P. Rall (previously a director of the National Institute of Environmental Health Sciences) responsible for the report also tells how the military gleaned uncovenanted

insight into the effects of poison gas from an incident in which a US military transport was sunk in the harbour of Bari, in southern Italy; 83 military personnel and nearly 1,000 civilians were killed.

That experiments of this kind should have been carried out at all is bad enough. That they should have been as poorly documented as the committee found is almost beyond belief. After exposure to mustard gas, even if there are no blisters, there may be a general reddening of the skin known as erythema. During the wartime exposure of people, this was taken as an index of damage done to service people, some of whom also complained of damage to their eyes or lungs. Even allowing for the wartime preoccupation of the military with the acute effects of poison gas on soldiers, it seems remarkable that so little was done, by medical examination, to understand the less than obvious effects of exposure, but that appears to have been the case.

The committee is rightly scathing about the whole affair, which has been a military secret since the end of the Second World War. Hapless participants in the trials were even sworn to secrecy before taking part, and seem loyally to have kept their silence for much longer than could reasonably have been expected. (In its preface, the committee pleads that the US government should even now acknowledge the bravery of these people, and honour those who still survive.) It also pleads for an understanding that military experiments with human subjects should in future be undertaken only under guidelines comparable with those proscribing biomedical research in the civilian sector. Inevitably, it concludes that many of the veterans who have complained of chronic illness as a consequence of their exposure have a case that needs answering; the committee would have been able to say more if it had been given access to all the facts. In short, it has described a scandal that calls for further digging. □

Clinton's honeymoon

President Bill Clinton deserves a longer respite from his most severe critics than he is getting.

WINNING the US presidency is a great prize, but it can also be a recipe for lying on a bed of nails. That may be President Bill Clinton's most vivid discovery so far. He seems to have been denied the period of indulgence usually offered to newly



installed presidents, who (with their advisers) face the formidable task of making more than 5,000 appointments to public offices before their governments can function properly. Part of Clinton's trouble is that he has uncharacteristically misjudged the politics of the city in which he now lives. Even so, it is not merely bad luck but carelessness that two successive candidates for the cabinet post of attorney-general should have had to withdraw when they were found to have employed aliens, in one case illegally.

But Clinton deserves good marks as well as poor ones for his first few weeks. It was sensible but also courageous to rescind President George Bush's prohibition of the use of fetal tissue in medical research, for example. That decision will not bring immediate benefits to patients, but will put the US medical community on an even footing with those elsewhere. The decision, at the same time, to get rid of restrictions on the use of federal funds for good works, in the United States and in developing countries, once judged tainted by abortion, will bring quicker benefits. Clinton has also made some good appointments, notably to the Department of Defense and the Office of Science and Technology Policy. His critics should acknowledge that.

The most serious weakness of the new administration is its ill-preparedness on issues left over from the Bush administration, international trade in particular. Negotiations on a new agreement under the General Agreement on Tariffs and Trade began more than six years ago. The administration's authority to sign a deal that will not then be picked over line by line by the US Congress expires on 1 March. Clinton might have come to office determined to cut a deal before the deadline, or alternatively to ask quickly for an extension of the authority. The first option has now been missed, but no decision has been made about the second. This puts in hazard an agreement that could do more to revitalize world trade, and thus to restore economic growth, than anything the US administration can accomplish on its own. Perhaps Clinton should have spent less time jogging and eating hamburgers between his Election and Inauguration Days. □

Cruel innovation

The plight of IBM and General Motors should be a lesson to successful technical companies everywhere.

WHATEVER has happened to International Business Machines Inc? Not so long ago, IBM was generally regarded as the most successful company in the world; now it is losing money at the rate of \$5 billion a year and, as a mark of its despair, has fired its chairman. And where now is General Motors, the giant of US industry since the 1930s, which during the Second World War was so technically competent that it could manufacture ships as well as motor cars and whose chairman in the 1950s went so far as to identify the interests of the United States at large with the well-being of his own company? GM, too, is in financial trouble and is busily closing manufacturing plants through-

out the United States. GM has also fired its chairman, but these public demonstrations that the companies recognize the seriousness of their problems are not in themselves guarantees of their survival.

The precipitate (but not necessarily permanent) decline of these huge enterprises is a great waste. That it seems to happen repeatedly does not blunt that frustrating sense. (What, for example, has become of the once-great steel companies of the United States, technical leaders in the early decades of this century?) Shareholders' funds are devalued when plants in which they have been invested are sold off cheaply or even dismantled to be sold as scrap. More important, vast amounts of intellectual work are squandered as able people's schemes for the future, in research laboratories and head offices, are put on the shelf. Still more important, careers are disrupted and, too often, prematurely terminated, with the permanent loss of technical skill.

That is why the problems at IBM and GM should be object lessons in risk-avoidance for other aspiring industrial giants. The most obvious explanations of what may have gone wrong do not apply. Size as such does not make successful companies dangerously inflexible (as much of the history of both IBM and GM shows). Nor does age. The world's successful pharmaceutical companies include a substantial proportion of the long-established. Nor, for that matter, is success itself a fatal encumbrance, as the case of the Boeing Aircraft Company amply illustrates. So what went wrong?

IBM's problem is the more easily understood. Since the late 1970s, it has been perplexed by an awkward conflict of interest — the fear that the growth of the personal computer business would undermine the chief source of its earlier fortune, the manufacture and sale of mainframe computers. It has sought to second-guess the market, but inconclusively and unsuccessfully. This is not, of course, the first time that a company's concern for its established business has persuaded it to be less than whole-hearted in pursuit of newer opportunities. In the event, IBM has shown that it had ample technical skill to follow both lines. It would have been able to do so successfully if it had split itself into two in the late 1970s, creating a small-computer business that could have fought the clone-makers soon to be snapping at its heels.

GM's weakness has been different. Technically excellent though it has always been, it was too slow to recognize that the technical excellence of motor cars designed elsewhere (notably in Japan) was a serious threat to its place even in the domestic market of the United States. (It is significant that GM's subsidiaries overseas, especially in Europe, have been more successful.) Mr Ross Perot, the presidential candidate last November, was briefly a member of GM's board who seems to have stirred up turbulence by advocating the need for change, then rejected but now being enforced. Complacency, of course, may be, but is not invariably, engendered by success. That is another danger against which the now-growing industrial giants should be on guard. □

Faraday Centres appear doomed after criticism by Lords' panel

London. A proposal for a network of new research and training institutions linking British academic research with industry has been knocked firmly on the head by a committee of the House of Lords. The committee claims in a report published last Monday (8 February) that the case for the so-called Faraday Centres "has not been made successfully". This criticism, taken together with a lack of enthusiasm from the Department of Trade and Industry (DTI), makes it highly unlikely that the proposal will ever get off the ground.

The idea of the centres, named after the nineteenth century physicist Michael Faraday, was largely the brainchild of Sir John Fairclough, formerly science adviser to Margaret Thatcher when she was prime minister. Fairclough chaired a working group set up by the Prince of Wales that last year recommended creation of a number of such bodies, based loosely on Germany's Fraunhofer Institutes. These are free-standing organizations, initially endowed by public funds with research funds provided in roughly equal amounts by government and industry.

As the working group envisaged it, the centres would provide facilities for both basic and applied research, as well as post-graduate training. And shortly before last year's general election the government announced a £2 million pilot programme to enable five existing industrial research organizations each to take on up to 20 graduate students (see *Nature* 355, 757; 1992).

Although these are operating successfully, the DTI, under heavy pressure to reduce its research budget, has so far balked at making any greater commitment. Michael Heseltine, president of the Board of Trade, told a meeting of the House of Lords Select Committee on Science and Technology in December that it was "not my intention to take the initiative any further".

The select committee's report pours more cold water on the idea. It says that such centres are not necessary to give scientists experience in industrially relevant research nor for promoting technology transfer and suggests that other, existing schemes would be more cost-effective. "Although we accept that universities have an important role to play in industrial innovation, this scheme is not what is wanted," says the committee's chairman, Lord Flowers, who as chairman of the Science Research Council in the early 1970s was responsible for initiating various programmes, such as the "teaching company" schemes, for increasing collaboration between industry and academic research.

The main enthusiasts for Faraday Cen-

tres, judging by witnesses who appeared before the Lords' committee, were research associations representing small and medium-sized enterprises (SMEs). These have been particularly enthusiastic about the idea that the government, through funding the cen-

IMAGE
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Lord Flowers says no.

tres, would be supporting applied research not targeted on specific projects.

Reactions from other quarters ranged from the agnostic to the sceptical. Large industrial corporations, which already have large research laboratories and good contacts with university scientists, said they

saw little need for a set of new institutions. And both the research councils and universities were concerned that the money would be taken out of their own budgets.

Among its counter-proposals, the Lords' committee suggests modifying the DTI's LINK scheme to enable it to support research themes, rather than just individual projects; expanding what it describes as the "very successful" teaching company scheme; and setting up regional and local "technology clubs" to help SMEs gain access to various forms of technology transfer.

Fairclough, a former research director of IBM who now works in the investment section of the investment bankers N. M. Rothschild, says that he disagrees with the committee's conclusion and that government funding for facilities allowing both industrial and academic researchers to conduct research "is a fundamental requirement". However, he points out that the original proposal was made before last year's elections and before the government decided to produce a white paper (policy document) on research and innovation. "I am more concerned to see what the government does in the white paper than to stick to the recommendations that we made *per se*, in a very different financial environment", he says.

David Dickson

French suspend child research pending review

Paris. A French research group has suspended its study into children born by artificial insemination using anonymous donors in accordance with the recommendation of a government inquiry into the matter. But the inquiry, prompted by allegations that the research contravened laws on medical confidentiality, data protection and informed consent (see *Nature* 361, 102; 1993), stopped short of recommending legal proceedings against the Paris laboratory of Pierre Roubertoux, an indication that a grey region exists between the needs of the law and those of research.

The inquiry by the Commission Nationale de l'Informatique et des Libertés ruled that researchers at the Centre National de la Recherche Scientifique/University of Paris V Laboratory of Genetics, Neurogenetics and Behaviour had not broken the laws on data protection because they had indexed the computerized data from the study using codes and not names. Nevertheless, the commission recommended that the research should be suspended for six months until uncertainties with respect to medical confidentiality and informed

consent are clarified.

The commission's major concern was that the researchers did not provide sufficient details of the experiment when asking the parents of the children for their consent. It recommended greater disclosure but conceded the relevance of the argument made by the researchers that full details would have "worried them [the parents] unnecessarily". The application of laws on informed consent to psychological research has been taken up by the national bioethics advisory committee, which is scheduled to give its opinion in a few months.

The inquiry judged that Sacha Geller, the operator of a sperm bank in Marseille, infringed existing laws by transmitting the names of the children to researchers. But it refrained from passing judgement on the question, pointing to a forthcoming parliamentary debate on a bioethics bill that would for the first time allow such information to be used for research purposes. With no legislation expected before the suspension expires, however, the legal status of research involving medical data remains in limbo.

Declan Butler

Court says US law applies to Antarctic waste cleanup

Washington. A US federal court ruling that an environmental law applies to Antarctica could seriously affect the way in which the US National Science Foundation (NSF) handles waste on the continent.

Environmental activists say the decision tells NSF "to look before you leap" by forcing it to follow a 23-year-old law that until now has applied only to activities on

NEPA does not apply to activities outside the United States but that NSF and other agencies must provide a similar review of any proposal that affects the environment under an executive order issued by President Jimmy Carter.

EDF says that requiring NSF to follow NEPA would lift the "shroud of secrecy" surrounding its actions in Antarctica and those of other federal agencies around the world. It argues that NSF did not adequately consider the impact on the environment of incineration when it decided in 1991 to end the open burning of waste at McMurdo and switch to incineration, and that incineration would generate heavy metals and other highly toxic pollutants. In May 1991 it brought suit against NSF, and three months later it appealed against a ruling by the district court that NEPA did not apply.

Photo by NSF

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A cargo ship waits outside McMurdo Station in Antarctica to transport a year's worth of boxed waste material back to the United States.

US soil. But NSF officials say that they are already "responsible stewards" of the environment and that the added regulatory review could disrupt fruitful international scientific cooperation by delaying some projects until all aspects of the environmental law are met.

On 29 January, the US Court of Appeals for the District of Columbia reversed a lower-court decision that NSF was not bound by the National Environmental Policy Act (NEPA). The suit was brought by the Environmental Defense Fund (EDF), which opposes the construction of an incinerator at McMurdo Station, the largest of three US research bases in Antarctica and the centre of US operations on the continent. The government has 45 days to ask the entire court of appeals to review the decision and 60 days to petition the US Supreme Court to take on the case. If the Clinton administration decides not to appeal, the lower court would then decide whether NSF followed the procedures spelled out under NEPA before building the incinerator.

NEPA requires US federal agencies to conduct an extensive and public review of any activity that might have a significant impact on the environment; it also gives dissatisfied parties the right to sue. US government lawyers have argued that

Government lawyers representing NSF told the court that NEPA could conflict with the laws of foreign countries and that its lengthy reviews could drive away potential research partners and prevent NSF from taking full advantage of scientific opportunities. They also argued that NEPA could dictate policies that might conflict with national security or other considerations.

NSF officials believe that the advocacy group's real purpose is to prevent the incineration of waste in Antarctica. Inadvertently, that goal has been partly achieved as technical problems have made it impossible for NSF to burn some food wastes in the new incinerator.

"We've had a problem dealing with [the water content in] frozen material", says Peter Wilkness, director of polar programmes for NSF. "Of course, the waste — mainly uneaten food — is not toxic; you can eat it. What's ironic is that we can't burn the stuff that wouldn't pollute."

During the past two years, NSF has shipped more than 50 tons of food wastes to New Zealand. But New Zealand is no longer willing to accept the waste, Wilkness says, and NSF is looking for another location. With the last ship due to leave McMurdo by the end of February before winter sets in, he says "we'll have to move fast".

Jeffrey Mervis

NEWS IN BRIEF

Washington. The American Chemical Society (ACS) has agreed to a cash settlement that ends a running battle with a local school system over the tax status of a successful subsidiary. As part of a negotiated settlement with the Columbus (Ohio) school system, Chemical Abstracts Services (CAS) will pay the schools \$2 million and an annual fee, \$275,000 this year and adjusted for inflation in future years.

The school system in Columbus, where CAS is located, is funded chiefly by local property taxes; it argues that CAS does not deserve its classification as a tax-exempt charity because the service benefits ACS's 140,000 members rather than the public. A second point of contention was the several million dollars each year that CAS contributes to ACS coffers, which the school board views as profit but which ACS uses to support the society's non-revenue programmes.

In June 1992, a local court ordered CAS to pay annual taxes of \$800,000, backdated to 1980 when the suit was filed. CAS appealed against the decision and at the same time persuaded state legislators to pass a law guaranteeing its tax-exempt status, precluding the school system from collecting taxes for 1993 and beyond. That statement, combined with a desire by new CAS leadership to put the issue to rest, provided a basis for the negotiated settlement reached last month.

Jenna Roberts

Washington. Energy Secretary Hazel O'Leary is the latest addition to a growing list of officials in the Clinton administration who are less than enthusiastic about the \$8.5-billion Superconducting Super Collider (SSC), on which her department this year is spending \$517 million. Meeting the media last week for the first time, O'Leary expressed considerably less zeal for the project than her predecessors, who took their cues from their bosses, presidents George Bush and Ronald Reagan. "I am in a balancing mode between our commitment to international cooperation on a high-energy physics experiment and the problem of reducing the federal deficit", she said. "And it's not going to be my call. But I can say that I'm not passionate about it."

Presidential science adviser John Gibbons was similarly equivocal when he appeared before Congress two weeks ago, and the new director of the Office of Management and Budget, Leon Panetta, was a sharp critic of the SSC as a member of Congress. Although the accelerator was brought up during a recent presidential retreat, President Bill Clinton has not as yet signalled whether he intends to abandon a campaign promise to support the project.

Jeffrey Mervis

CERN expels four Serbs to meet UN sanctions

Munich. CERN, the European Laboratory of Particle Physics, has been accused of breaking UN sanctions by allowing a small team of Serbian scientists to continue their research for a TESLA cyclotron being built in Belgrade. Following reports in the Swiss press last Sunday that criticized its conduct, CERN has told the four scientists that they must leave by the end of the month. The expulsion is the first in the 38-year history of the laboratory.

It is eight months since the United Nations imposed economic and cultural sanctions on Serbia, and it is not clear how the scientists managed to escape attention for so long. CERN says officially that it was hoped that the scientists would leave of their own accord and that they were given ample time to do so. But other interpretations suggest that the scientists were allowed to continue working because of a view that science and politics should be kept separate, and that they managed to avoid detection by keeping to themselves.

The former Yugoslavia was a founding member of CERN and, although leaving the organization in 1961, it has maintained a degree of scientific cooperation through its status as an 'observer'. But that relationship was believed to have ended this past summer, when Carlo Rubbia, CERN's director general, issued a written statement on 10 June that CERN would "fully comply" with the UN resolution prohibiting any type of interaction with Serbia. Since then, according to laboratory officials, CERN has taken "all the necessary measures to run down the activities of cooperation". Computer networks were shut down, the exchange of scientific materials was stopped and visits to Serbia by CERN personnel were prohibited. Nonetheless, the team of four scientists continued to work at CERN's laboratory in Geneva.

The national interdisciplinary laboratory at Vinča decided in the mid-1980s to develop the US\$16 million TESLA cyclotron as a research facility for local scientists and as a user facility and source of radioisotopes for medical purposes throughout the Balkans. In November 1990, CERN signed a collaborative agreement to provide advice on radiofrequency systems for TESLA, and eight scientists from Vinča were given cards allowing them access to CERN as unpaid visiting scientists. Four set up a laboratory to work on their model for the radiofrequency equipment, and since last summer they have had minimal contact with their home institute.

CERN no longer used cyclotrons, but officials arranged for a recently retired ex-

pert on cyclotron radiofrequency to continue working as an adviser to the group. This collaboration continues, supplemented as needed with impromptu advice from other CERN scientists.

Bosko Bojović, a Serbian research fellow who started work at CERN in January 1991, says that he and his colleagues are unhappy about being sent home before their model could be tested. Once they lose their CERN cards, he says, their visas will be rescinded and they will have to return to



This building in Belgrade will house the TESLA cyclotron.

Belgrade. "We will certainly not be able to carry on our work at home," he adds.

The Serbian scientists say that they were allowed to continue their work because the TESLA cyclotron is intended to be used primarily for medical research and was therefore exempt from sanctions. CERN officials deny this and say that the work should have been stopped much earlier.

The laboratory has a long tradition of scientific exchanges. During the depths of the Cold War, scientists from the Soviet Union and eastern Europe were welcomed at CERN. Guy Hentsch of Rubbia's office says that CERN "wanted to act in a decent way and give them time to leave spontaneously". It was regrettable, he says, that they eventually had to be told they were no longer welcome.

But CERN's role in the incident is unclear. A report in Switzerland's populist newspaper *La Suisse*, which wrongly suggested that the cyclotron could be used for military purposes, portrayed Hentsch as being sympathetic to the plight of the group by quoting him as saying that it would be cruel "to break up a phase of their project just before it was finished". The Serbian scientists say they were given neither an official nor an unofficial directive about leaving CERN and were always made to feel welcome, at least by their immediate colleagues. The Vinča Institute says that it had understood that their colleagues would not actually be thrown out unless Serbia increased its role in the war.

Once the Serbian group returns home, any hope of building the cyclotron, whose housing complex in Belgrade is already complete, depends upon the sanctions being lifted. Back in Geneva, CERN will need to find a more convincing way of answering charges that it ignored UN sanctions if it wants to lay to rest an unpleasant incident.

Alison Abbott

Monthly Nature's launch in Moscow

London. A trial edition of a monthly issue of *Nature* will be produced in Moscow next Monday and distributed to some hundreds of people at a reception at the People's House of Science as well as more generally. The trial issue will contain contributions appearing in *Nature* in three issues towards the end of 1992. The intention is that this first issue will be followed, no later than April, by the regular publication of a selection of contributions from the previous month's issues of the standard edition of *Nature*.

The new periodical, called *Monthly Nature*, will be in English to avoid the costs and delays of translation. The selection of each month's contents will be made in Moscow by a committee of Russian scientists. It is intended that the new journal will circulate not only in Russia but also in the other republics of the Commonwealth of Independent States.

The production of the trial edition has

been arranged by Yury Eldyshev and Carl Levitin, previously joint managing editors of the Academy of Sciences journal *Priroda* (which means 'nature'), who will also be employed by a *Nature* enterprise being established in Moscow to manage the regular monthly production. The new arrangement will not disturb the long-standing collaboration between *Nature* and *Priroda*.

The immediate objective of *Monthly Nature* is to make the essence of each month's *Nature* accessible to and affordable by scientists in the republics of the Commonwealth of Independent States. The management of *Nature* sees this as an interim goal on the way to the publication of the regular weekly edition somewhere in the territory where the ruble is the standard currency. The standard weekly edition of *Nature* is already produced once a week in Virginia (United States), Tokyo (Japan) and Beijing (China) as well as in Britain.

Japan begins processing data for genome programme

Tokyo. An important element in Japan's efforts to process data from the human genome project was last week opened to outside users. The Human Genome Centre of Tokyo University's Institute of Medical Science is one of two central dataprocessing facilities — the other being the Supercomputer Laboratory of Kyoto University — in a rapidly expanding computer network for handling genome data.

The centre, located in a new, two-storey building at the back of the Institute of Medical Science, is not as grand as what was described in a 1990 plan by a committee of nearly 70 scientists headed by Kenichi Matsubara of Osaka University. Its recommendation for eight laboratories and a permanent staff of 36 was reduced to three laboratories by the Ministry of Education, Science and Culture, which runs Japan's national universities (see *Nature* 349: 360; 1991).

Two are operating: the Laboratory of Genome Database, which last week opened its computer system to outside users, and the Laboratory of Genome Structure Analysis. The third, the Laboratory of DNA Information Analysis, will begin work in April. About 15 scientists, including students, are in the building, with some members of the genome structure analysis laboratory working in temporary quarters elsewhere. Plans for a supercomputer were dropped after a 1990 US-Japan trade agreement severely limited the number of supercomputers the ministry can buy each year and the centre decided not to wait in the queue (see below).

Nevertheless, there is an impressive array of hardware on hand, most of it from the United States and leased at an annual cost of ¥300 million (\$2.4 million). There is a nCUBE2 parallel computer with 64 processors and a CM-5 parallel computer with 32

processors to develop parallel algorithms for handling genome data, powerful database servers (SUN SPARCstation 690MP) and computation servers (IRIS Crimson VGXT), as well as numerous graphics and engineering workstations. The only large piece of hardware from Japan is the Hitachi HITAC M-860/160 file server.

The genome centre is joined by a high-capacity (1.5 megabit per second) computer link to the faculty of science on the main campus of Tokyo University on the other side of the city. From there, the centre's computers can tap into the rapidly expanding Todai International Science Network (see *Nature* 356, 550; 1992), which provides access to the US network Internet and US databases, such as the Genome Data Base (GDB) and GenBank. The centre's scientists have developed their own integrated database system called HyperGenome to retrieve information on genetic maps stored in GDB and sequence data stored in GenBank; this database is available free to outside users.

The centre's wide-area network, called "GenomeNet", has high-capacity links to five other centres in Japan. GenomeNet is also linked to another high-capacity network, the Widely Integrated Distributed Environment (WIDE), which is used mainly by researchers developing computer networks but also provides university scientists with access to overseas networks.

The supercomputer laboratory at the Institute for Chemical Research (ICR) in Kyoto, the other main centre of GenomeNet, complements the Tokyo centre; Kyoto offers powerful computation capability and Tokyo will develop advanced databases and algorithms with its parallel computers. Minoru Kanehisa, head of the genome database laboratory and a professor at ICR,

Researchers like US products

Tokyo. A striking feature of the new Human Genome Centre is how much of its equipment is not Japanese. Not only are most of the computers US-made (see left), but also a pilot semi-automatic sequencing system in the Laboratory of Genome Structure Analysis consists largely of US and European machines.

Japan's Science and Technology Agency has invested several million dollars over the past ten years in automatic DNA sequencing machines in collaboration with Japanese companies such as Seiko and Hitachi (see *Nature* 351, 593; 1991). But given a choice, it seems that Japan's genome researchers prefer machines made by Applied Biosystems Inc. of California and Pharmacia of Sweden.

The pilot system at the centre has a much smaller capacity than the system run by Craig Venter, who last summer left the US National Institutes of Health (NIH) to run a new, private sequencing company. But the laboratory hopes to build a much larger system if the centre gets the money to move to a larger building. **D.S.**

divides his time between the two facilities.

Kanehisa expects the number of Japanese users eventually to reach a thousand, most of them at universities and national laboratories. But fewer than two dozen tapped into the facility on opening day.

A lack of money has already delayed one important component of the network. In 1991, the Science and Technology Agency, a rival of the education ministry, applied for funds for the fiscal year beginning 1 April 1992 to establish a high-capacity international computer link between the Japan Information Centre of Science and Technology (JICST) in Tokyo and GDB at Johns Hopkins University in the United States. Under the plan, JICST would be linked to the genome centre.

But the Ministry of Finance rejected the request for administrative reasons, and later awarded to the agency's Institute of Physical and Chemical Research only ¥112 million of the initial request of ¥280 million. One consequence is that the international link to GDB cannot begin before January 1994.

Sanzo Miyazawa of Gumma University, who is helping to establish the JICST network, says that JICST will be connected to the genome centre and other networks and will serve private companies and national laboratories, while the genome centre will be used mainly by university researchers. But how that will work in practice remains unclear. **David Swinbanks**

Trade agreement backfires

Tokyo. The 1990 trade agreement between the United States and Japan, rather than increasing sales of US supercomputers to Japan's universities and national institutes as intended, has made it harder for Japanese institutions to buy supercomputers from any source.

Before the agreement, universities were buying about five supercomputers a year at a large discount from Japanese companies. But the agreement eliminated the discounts, and the Ministry of Education, Science and Culture now approves the purchase of only two or three supercomputers a year. As a result, universities are having to wait several years, or, as in the case of the Human Genome Centre, buy less powerful machines.

Universities also hesitate to buy supercomputers for fear of offending a competitor. For example, when the National Institute of Fusion Science selected a NEC supercomputer, Cray Research of the United States filed a formal complaint based on allegations that NEC exaggerated the machine's performance (see *Nature* 358, 265; 1992).

Since the agreement, Cray has sold only three supercomputers to Japan's public sector, while its sales to Japan's private sector over the same period are much larger. **D.S.**

Britain finds way round impact of falling pound

London. Fears that Britain might have to reduce significantly its participation in international scientific projects as a result of last autumn's collapse of the pound have lifted, at least for now, after negotiations to reduce the impact of currency fluctuations.

Shortly before Christmas, Sir Mark Richmond, the chairman of the Science and Engineering Research Council (SERC), was warning that catastrophe could follow if Britain was required to meet the full increased costs of its membership in organizations such as the European Space Agency (ESA) and the European Laboratory for Particle Physics (CERN). At one point, Richmond even cast doubt on continued membership in these organizations, paid for in European Currency Units (ECUs) and Swiss francs respectively.

However, negotiations have reduced the impact of recent changes in the exchange rate. Space officials, facing an extra charge of £20 million, have negotiated the increase down to £7 million, and Britain's annual subscription to CERN actually has been reduced by £3 million from the £57 million previously anticipated.

The ESA negotiations centred on the procedure under which countries either top up their membership subscriptions or become eligible for refunds if exchange rates for converting national currencies into ECUs fluctuate widely after having been set at the beginning of the financial year. British ministers warned last November that, if this procedure were strictly applied, Britain would no longer be able to help finance the laboratory module being developed by ESA for the US space station.

With both Spain and Italy suffering from similar currency devaluations and Germany pushing hard for a reduction in the agency's overall budget to help pay for the country's reunification, ESA's governing board agreed on a new adjustment formula. Under this, member states that have suffered a devaluation in their currency relative to the ECU will pay only 35 per cent of the resulting extra costs. And those whose currencies have increased in value (primarily Germany and France) have agreed to accept only 50 per cent of the refunds to which they would otherwise have been entitled.

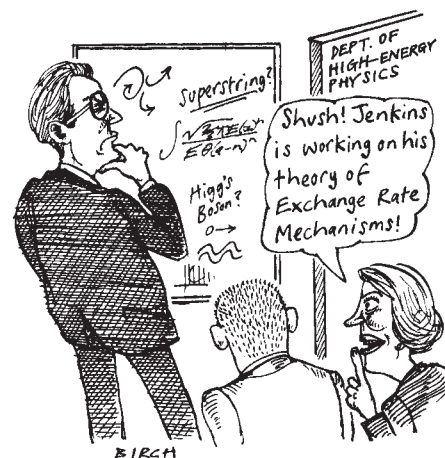
ESA must provide whatever additional money is needed, increasing the pressure to trim some activities. But British officials are relieved at the outcome. "Finding the extra £7 million out of an annual ESA subscription of about £110 million will still be difficult. But it will certainly be much easier than finding £20 million", said one.

A lower subscription price to CERN is the result of three factors. One was Britain's agreement last summer to make an advance payment on its 1993 subscription to help the organization with a temporary financial problem. The second was the financial implications of devaluation itself, which will reduce Britain's subscription to CERN — based on an assessment of "net national income" — from 15.2 per cent of the laboratory's total budget in 1992 to 13.5 per cent in 1993.

The third factor was the decision last December by the CERN council, again primarily under pressure from Germany, to reduce the laboratory's 1993 budget by 3 per cent (or \$10 million). No decisions have been made on how these cuts will be achieved, although the installation of new equipment and perhaps certain experiments are expected to be delayed.

Half of Britain's savings from its reduced subscription will be returned to the SERC's central fund and redistributed according to the council's scientific priorities. The other half will be returned to the nuclear physics board and reallocated to domestic research projects.

Nuclear physics, however, is not yet out of the woods. The SERC has offered up three programmes in response to a request from the Advisory Board for the Research Councils (ABRC) for suggested ways to absorb hypothetical cuts of 2, 4 or 6 per cent in next year's budget.



One would be to abandon all domestic research into nuclear structure, a move that the council prefers to opting out of CERN's plans for a new particle accelerator, the Large Hadron Collider. The other two programmes are British participation in ESA's X-ray Multi-Mirror space mission and the Vulcan high-energy laser facility at the Rutherford Appleton Laboratory.

Physicists feel that they have been unfairly singled out by the SERC as potential victims of the ABRC budget knife. The Institute of Physics has written to Richmond in protest, pointing out that a special review carried out for the SERC two years ago concluded that nuclear structure research in Britain "is of high quality and enjoys an international reputation". It has asked for an assurance that programmes in other disciplines be subject to the same degree of scrutiny before decisions are made about what programmes might be cut.

David Dickson

Russia sets up system for individual grants

Moscow. The Russian Foundation for Fundamental Research has begun accepting proposals for the first round of peer-reviewed research grants to individuals. But a deadline of 15 March for the distribution of the first quarter of its annual budget of 7.7 billion rubles (US\$15 million) has forced the foundation to move more quickly than it would like, jeopardizing its promise to rely on experts in the various fields and to bypass the bureaucrats who controlled funding in the former Soviet Union.

The foundation, using government funds, hopes to give grants of up to 5 million rubles to individuals or small research teams. About 60 per cent will be spent directly on research, with 7.5 per cent for international travel and collaboration and 7.5 per cent to publish results. The remaining 25 per cent would go to the institution for overhead costs. The foundation is expected to make 200–300 grants initially from a pool of

several thousand applicants.

Meeting recently for the first time, the foundation's board of directors, composed primarily of the heads of several well-respected institutes, defined six broad areas of support. Each area will have a council of experts of a dozen or so members reviewing proposals, although there is no guarantee that every proposal will be judged by an expert in that field.

The foundation is headed by Andrei Gonchar, vice president of the Russian Academy of Sciences. Gonchar is heartened by the existence of the International Science Foundation, created last autumn by US businessman George Soros to distribute US\$100 million in grants to individual researchers throughout the former Soviet Union. Gonchar hopes that the Soros foundation will be a model for the Russian foundation to follow and perhaps even a healthy competitor in seeking out the best applicants.

Vladimir Pokrovsky

UK blood centres could play major role in gene therapy

London. Britain's public blood transfusion services could eventually evolve into a national "gene replacement" service under proposals tabled last week by a group of prominent medical scientists. The group suggests that, as gene therapy becomes more widely practised, the services' experience in collecting blood from donors and delivering it to hospitals could be adapted to the task of collecting cells and organizing their genetic manipulation before transplantation into patients.

The Scottish National Blood Transfusion Service (SNBTS) is already taking a small step in this direction. Researchers at its centre in Edinburgh will work with local university and hospital groups that are preparing gene-therapy experiments.

No such formal steps have yet been taken in England, where a national agency begins work on 1 April. However, its director, Harold Gunsen, said last week that involvement in gene therapy could become an important activity for blood transfusion services in the light of growing interest in synthetic blood substitutes produced by the biotechnology industry and declining demand for some conventional blood products. "Who knows, in 20 years this may be the main work of the transfusion centres", says Gunsen.

The idea of involving the transfusion services comes from a task force on gene therapy and transplantation set up by the Advisory Council on Science and Technology as part of a broader study of medical research and health (see *Nature* 361, 385; 1993). Chaired by Keith Peters, professor of medicine at the University of Cambridge, the task force was asked to look for ways of

linking genetic research to social applications. It says that although a number of groups in Britain are likely to begin gene-therapy experiments over the next few years, there will be several requirements that are "almost certainly" beyond the scope of most research laboratories in medical schools and research institutes.

In particular, monitoring for safety and "good manufacturing practice" will require expertise in handling cells and a sophisticated knowledge of retrovirology and other virus vectors, the task force says. "We believe that the infrastructures and some technical expertise is already available" within the transfusion services, it adds, suggesting that one or two centres should receive support for work in gene therapy and transplantation.

John Cash, medical and scientific director of the Scottish service, says that the task force's proposals "fit in well with what we are planning". He points out that the service already has expertise in molecular biology, and is involved in collecting stem cells, for example for use in leukaemia treatment. Preparing cells for gene therapy — perhaps eventually including putting genes into cells — would therefore be a logical extension.

One advantage of involving the transfusion service in gene therapy is its good reputation. "People are used to the idea that the service handles products which are of human origin, and are then given to other patients," Peters says, adding that the service may help gene therapy to escape the consequences of a growing public distrust of scientific experts.

David Dickson

US biotechnology groups select new executive

Washington. Carl Feldbaum, 49, a lawyer with experience working for both Congress and the executive branch, has been chosen to head the new Biotechnology Industry Organization (BIO), the trade organization representing the US biotechnology industry after the merger next July of the two leading trade associations — the Association of Biotechnology Companies (ABC) and the Industrial Biotechnology Association (IBA).

Steven Duzan, chairman and chief executive officer of Immunex Corporation and chairman of IBA, says that Feldbaum was given highest marks for his "tremendous capacity to absorb and to talk about technical information". For the past four years, Feldbaum was chief of staff for Senator Arlen Specter (Republican, Pennsylvania) and previously served both Democratic and Republican presidents as an assistant to the Secretary of Energy and as inspector general for defence intelligence in the US Department of Defense.

Richard Godown, president of IBA, and William Small, executive director of ABC, will stay on as senior officials in the new trade organization.

Diane Gershon



Carl Feldbaum

Chinese seize special issue of journal

Washington. The Chinese government has muzzled a 72-year-old Chinese physicist and long-time human rights activist by confiscating all 20,000 copies of a scientific journal that devoted a recent issue to political reform in China. Western-style economic reforms cannot succeed without democratization and political freedom, wrote Xu Liangying in the October 1992 issue of *Future and Development*, an official publication of the China Association of Science and Technology.

"If Xu were 27 instead of 72 he would probably be in jail", says Richard Dicker of the New York-based Committee to End the Chinese Gulag, "but it would be a serious scandal for the government to arrest someone of his age". Xu sharply criticized the principles adopted by the recent 14th Party Congress in China in an article entitled "Without democracy there will be no reform", condemning government leaders for rejecting calls for reform while following "the nonsense and drivel uttered by liars, hoodlums and prostitutes". **Jeffrey Mervis**

Meetings on British science

Nature is organizing two one-day public meetings in Edinburgh and London to discuss proposals for the forthcoming white paper on the organization of British science.

On **Friday 19 February**, there will be a meeting in **Edinburgh**, jointly sponsored by the Edinburgh International Science Festival. The speakers will be Professor David Glover (University of Dundee), Sir Graham Hills, Professor J Maxwell Irvine (University of Aberdeen) and Professor David Wallace (University of Edinburgh). The meeting will be at the Queen Mother Conference Centre, Royal College of Physicians, 9 Queen Street, Edinburgh, and will start at 9.30 a.m. Admission will be by ticket, free of charge, obtainable from Bruce Durie, Edinburgh International Science Festival, 1 Broughton Market, Edinburgh EN3 6NU.

On **Friday 19 March**, there will be a meeting in **London** at the Royal Society, 6 Carlton House Terrace, SW1. The speakers will include Sir Mark Richmond (Chairman, Science and Engineering Research Council) and Professor Michael Brady (University of Oxford), and the meeting will start at 9.30 a.m. The other speakers will be announced later. Admission will be by ticket, free of charge, obtainable from Mary Sheehan, *Nature*, 4 Little Essex Street, London WC2R 3LF.

Bad times force the Swiss to reduce spending plans

Basel. The Swiss federal government has for the second year running reneged on a budgetary promise to researchers, leading to cuts so severe that some departments may have to close. The reductions are part of the government's attempt to reduce an annual deficit as high as SFr5 billion (US\$3.5 billion).

The Swiss National Science Foundation (NSF), which awards grants to university scientists, was told recently that the increase of 9.5 per cent it was promised in 1991 has been cut in half as a result of a decision by the Swiss parliament to trim federal spending by 10 per cent. The foundation is still reeling from a decision last year by parliament to reduce by half its budget for a new strategic research programme.

Universities will fare even worse in the retrenchment. Although the federal government provides only 16 per cent of their budget (individual cantons provide the rest), universities were promised an increase of 23 per cent in 1993 after a 1991 report showed that the government had fallen behind on its commitment to university research. But given its current problems, the government this year will offer an increase of only 3 per cent (see chart), a figure that barely matches

the rate of inflation.

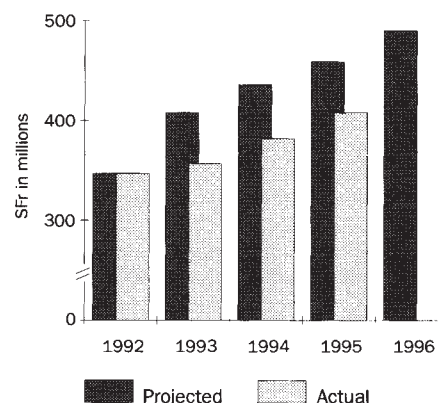
This decision will force universities to eliminate posts, according to Nivardo Ischi, general secretary of the body that represents Swiss university administrators. He estimates that career opportunities for young researchers will shrink by half between 1992 and 1995.

Cantons are also feeling the pinch. For example, the Basel government has asked its university to propose ways to reduce spending by 14 per cent, and the administration has suggested closing its teaching programmes for preclinical medical students and also several departments. The University of Bern must save SFr8 million by 1996, and is expected to lose half a dozen professors from a 80-member science faculty.

Reduced federal spending also affects independent research institutes such as the Swiss Institute for Experimental Cancer Research in Lausanne. The institute will get SFr1 million less this year than the SFr6.6 million it expected from federal sources, forcing it to dip into its capital to cover operating costs. "We fear that good researchers may leave us because they feel the institute has no future", says its director, Bernhard

Promises, promises

Government spending on university research



Hirt, who has been unable to recruit replacements for a retrovirus research team that left last year.

As the situation in universities worsens, the number of researchers asking the Swiss NSF for grants increased by 15 per cent between 1990 and 1991. In response, the NSF plans to distribute the reductions evenly across all areas, postponing or cancelling many new projects.

However, general secretary Peter Fricker sees a silver lining in the latest cloud. "I don't fear a brain drain", he says, "because the situation abroad is certainly no better."

Oliver Klaffke

Germany asked to increase spending on universities

Munich. Germany's influential science council, the Wissenschaftsrat, wants the government to increase spending on university research and on institutions in the eastern states.

The Deutsches Forschungsgemeinschaft (DFG), the largest sponsor of university research, for the first time is rejecting a majority of grant applications because its budget has not kept pace with increased demand from a growing scientific community. Germany remains among the leading sponsors of research and development in the world, with spending as a percentage of gross domestic product (GDP) rising 0.4 points to 2.81 per cent during the 1980s. But the science council, in a recent report, argues that universities are not getting their fair share.

Total spending on university-based research has fallen since 1975 from 1.32 per cent to 1.12 per cent of GDP. Germany's recent economic boom, combined with a 45-year-old law guaranteeing higher education for all high school graduates, has contributed to a near doubling of the annual number of university graduates during that period, from 841,000 to 1,582,000. That growth has led to an increased demand for research funds (more

than 10,000 applications in 1991).

The success rate has fallen steadily for the past two decades, from more than 70 per cent in the 1970s to 60 per cent in 1985 and to 43.3 per cent last autumn. For the first time, the DFG finds itself unable to fund even projects assigned top grades. This is unacceptable, says the science council, which believes that the DFG, to function properly, should fund at least half of the applications.

An agreement in 1990 between publicly funded research organizations and the federal and regional governments pegged the growth in spending to five per cent a year for five years. This 'five-times-five' agreement ran into trouble last year (see *Nature* 357, 182; 1992) when wage settlements of 5.4 per cent were granted. Despite that, Wilhelm Krull of the science council would like the governments to extend the agreement to the end of the century so that grant-giving bodies can plan a long-range strategy. In the face of a faltering economy, it argues, support for research should be maintained to ensure that the country holds its competitive edge.

The financial strain on research funding has been increased since reunification in October 1990, when the DFG was told to make research in eastern universities on a

par with western levels. In 1991 it was given DM90 million (US\$55 million) for that purpose, but the amount has been insufficient. The DFG has long argued that universities in eastern Germany are so old and badly maintained that they cannot offer the most fundamental of facilities for modern research. "We can give a university a beautiful new centrifuge or Coulter counter", says Christoph Schneider of the DFG, "but then we find that the lab floor, with unrepaired holes giving a view to the storey below, simply cannot bear the weight".

The science council believes that research facilities must be rebuilt before standards can be raised, but it does not want the DFG's grant money to be diverted from research. Instead, it says, the federal and regional governments should meet their joint responsibility for rebuilding the infrastructure.

The council is composed of representatives from federal and regional governments, including those from finance and planning departments, as well as scientists. The new report must now pass through several levels of political assessment. Although the council has a good record of success with its recommendations, that may be more difficult this time around as Germany tries to revive a troubled economy. **Alison Abbott**

Suppressing dissent?

SIR — The debate (F. M. Menger and A. Haim, *Nature* **359**, 666; 1992; R. Breslow, **360**, 23; 1992; R. L. Schower **360**, 506; 1992) concerning the merits and refereeing of a communication to a leading periodical (*Journal of the American Chemical Society* or *JACS*) deserves, in my opinion, further brief comment.

First, it has not been explicitly recognized that communications (or letters) to leading journals (for the physical and biological sciences, *Physical Review Letters*, *Science*, *Nature* and *JACS* is a sensible if incomplete listing) are considered as a working definition of leading research in these areas and as a starting point for further studies. As such, communications (or letters) have, in addition to the usual attention and refereeing, a special obligation to be reflective, accurate and complete.

Second, of the four periodicals listed above, *JACS* only does not provide a forum (correspondence or comments) for an open discussion of possible errors in analysis or interpretation. Regardless of the merits of the communication in question or the subsequent correspondence, the deliberation offered in *Nature* should have rightfully appeared in *JACS*; it would be sensible for that periodical immediately to take steps to provide such a medium for exchange of views.

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SIR — Surely the time has come to institute an international code of ethics for referees? Some societies (including the American Chemical Society) have their own 'ethical guidelines', and most journals have 'instructions for authors'. But would it not be possible for editors of major scientific journals to agree to a common set of principles that would serve as general guidelines for referees, irrespective of the scientific research field?

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SIR — I have read with interest the article by Haim and Menger and the reply by Breslow. I am surprised by what seem to be attempts at suppressing dissent; an action that becomes neither the people involved nor their positions. In pleasant contrast is the case of Pauling

(*Proc. natn. Acad. Sci. U. S. A.* **86**, 8595–8599; 1989) encouraging simultaneous publication of the criticism by Bancel *et al.* (*Proc. natn. Acad. Sci. U. S. A.* **86**, 8600–8601; 1989) of his own work.

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UK research

SIR — The numerical value of many statistical functions changes with sample class boundaries. Thus the improvement in British university research that David Dickson (*Nature* **360**, 700; 1992) inferred from the figures released by the Universities Funding Council may be real but it may be an artefact of departmental closures, mergers and selective inclusion of staff in departmental figures. It is likely that the three-year period since the previous assessment is too short to measure any real improvement in active research, given the time-lags between grant applications and conclusion of research and between research and final publication. Much of the documented improvement could reflect a greater time spent in writing papers and grant applications with a consequent decrease in the time employed in active research. If so, the data may conceal an actual decline in the amount and quality of current research. The view that the quality of UK university science research has improved is at odds with the recently reported decline in the national science citation record. In my opinion, we currently have insufficient data to draw reliable conclusions on developments in UK science research.

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Not in our *Nature*

SIR — Several *Nature* issues have contained advertisements mentioning some of the important scientific discoveries originally published in *Nature*, among them Darwin's theory of natural selection, the first rocket, Watson and Crick's DNA double helix, the production of monoclonal antibodies and male development of chromosomally female mice.

I understand that science is a commercial activity and that scientific journals, keystones in the edifice of science, must compete to attract readers. But scientific

journals, like scientific researchers, can make mistakes, and it is the sum of all these successes and failures that determines the general course of the advance of science. As well as publicizing the most important scientific discoveries published in *Nature*, you should also admit to the errors you have made in rejecting important papers that went on to influence and shape their disciplines. In some cases their authors received the Nobel prize.

(1) In 1981, *Nature* rejected a paper by the British biochemist Robert H. Michell on signalling reaction by hormones. This paper has since been cited more than 1,800 times.

(2) In June 1937, *Nature* rejected Hans Krebs's letter describing the citric acid cycle. Krebs won the 1953 Nobel prize in physiology or medicine for this discovery.

(3) *Nature* initially rejected a paper on work for which Harmut Michel won the 1988 Nobel prize for chemistry; it has been identified by the Institute for Scientific Information as a core document and widely cited.

(4) A paper by Michael J. Berridge, rejected in 1983 by *Nature*, ranks at number 275 in a list of the most-cited papers of all time. It has been cited more than 1,900 times.

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Alexander Bain

SIR — It was good to see Alexander Bain mentioned in the commemorative Commentary by J. L. Heilbron and W. F. Bynum (*Nature* **361**, 9–12; 1993). However, his 1843 patent was of more significance than just being a description of his "premature" invention of a telegraphic fax machine. It was almost certainly the first publication proposing the important principle of scanning an image to enable it to be transmitted electrically. As such it was the precursor of many important scientific techniques as well as, of course, television technology.

Incidentally Bain died, at the age of 67 and in near penury, in 1877 not 1903.

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■ The Alexander Bain who died in 1903 was the philosopher and logician.

Various readers have pointed out *Nature's* error in identifying the subject of Gillray's cartoon on the cover of the 7 January 1993 issue as Lavoisier rather than Humphrey Davy. □

Universal Darwinism

SIR — G. A. Dover¹ dismisses Dawkins's claim for Universal Darwinism² by saying that "there is nothing rational or law-like about biological organization and the processes that gave rise to it, given that there are no predictable regularities of events on a par with physical phenomena". Biologists are indeed driven by the astonishing diversity of life forms and fascinated by "unpredictable organisms"¹. But this must not preclude biologists from finding general principles as physicists do. Trying to focus on (some) individual atom trajectories will not lead one to the laws describing the statistical behaviour of large sets of atoms when some ideal conditions are met. Drawing on the same analogy with the properties of gases, an early commentator, C. S. Peirce, noted that "Darwin, though unable to say what operation of variation and natural selection in any individual case may be, demonstrates that in the long run they will, or would, adapt animals to their circumstances". When a stone slides down a rugged slope, the law of gravity tells us that it will go down, not where it will end up. Science looks not for complete descriptions of facts but for abstracted, testable, universal laws. Biology has at least one such law, evolution by natural selection, which states that a trait distribution will inevitably change from generation to generation whenever some conditions are met: the trait affects reproductive success, is (at least partly) heritable and varies among individuals. Besides species, this law applies to test-tube RNA or computer programs. Einstein published his ideas years before they were actually tested. Biologists would gain from such an open attitude towards theoretical work³. The complexity of biological systems should not prevent us from making universal statements on how this complexity came about. One such statement is that factors other than selection (Lamarckism, mutationism, for example) have only secondary roles. Left alone, those nonselective factors could never produce evolution of organized complexity as we know it, as Dawkins has forcefully demonstrated in his writings, but natural selection alone probably could.

Vincent Bauchau

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SIR — "There is," avers Gabriel A. Dover¹, "nothing rational or law-like about biological organization and the process that gave rise to it, given that there are no predictable regularities of

events on a par with physical phenomena." Thus he refutes Richard Dawkins.

Dawkins had written that Darwinian natural selection must be a necessary feature of life anywhere in the Universe (*Nature* 360, 25; 1992); it has to be the case, *a priori*, and empirical evidence, when found, can only confirm this.

I cannot see how Dawkins can be faulted. Let us ignore the one case exemplar we have. If life exists, existed or ever will exist somewhere at some time in the Universe, carbon-based or otherwise, the forms most in harmony with their environment will be those that tend to prosper and survive. If there is any biologist alive, including Dover, who does not hold to this axiomatically, then his arguments against this proposition will be interesting to learn. (Something based on probability theory perhaps?)

Biological organization and processes are and must be as rational and law-like as any other physical phenomena, life being one form of such phenomena and not distinct from them in kind but only

in degree. Life's complexity, variability, evolution and general unpredictability are not in themselves any evidence of any irrational or lawless quality. Individual forms will vary to suit individual circumstances but the fact that we are unable to predict "regularities of events" in any precise way has nothing to do with the nature of "Universal Darwinism", any more than the fact that we do not know the chemical constituents and other particulars of the farthest quasar — one indeed that we shall never see or even be aware of — has to do with the universality of the law of gravity in its case. Life may not be the same thing as gravity, but that does not mean it is not governed by universal law, one of these being the origin, evolution, and survival (or lack of it) by means of natural selection.

To hold otherwise is to abandon science.

Ralph Estling

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1. Dover, G. A. *Nature* 360, 505 (1992).

2. Dawkins, R. *Nature* 360, 25–26 (1992).

3. Huzzag, V. A. & Infante, J. P. *Nature* 338, 109 (1989).

Selling the future

SIR — Reading about the "government proposal to sell off research institutes" (*Nature* 360, 614; 1992), I quickly checked the date to see whether I was in possession of an advance copy of the 1 April issue. Having ascertained that the proposal, to sell off the Medical Research Council's Laboratory of Molecular Biology (LMB) in Cambridge and the National Institute for Medical Research at Mill Hill, was not an April fool's joke, I concluded that the designated fools were other than your readers.

The institutions in question have made great contributions to international science due, at least in part, to their ability to concentrate on long-term, important problems, rather than on the quarterly "bottom line". The proposal to trust them to the tender vagaries of the marketplace will surely astound those familiar with these institutions, as well as with the workings of science. The United States had to develop special legislation for industry to fund even life-saving research that is applied, but has only modest or no-profit potential. This, the case of the 'orphan drugs' used to treat rare diseases, is but one example of the futility of relying on the private sector for basic research. One can also point to the reduction in research and development associated with declines in the business cycle, as illustrated by the recent cutbacks of such paragons of private research as Bell Laboratories.

Does the proposal to separate "curiosity-driven" from "mission-oriented" research imply that some scientists are not curious enough to seek cures for cancer, atherosclerosis or HIV infection? The very important mission-oriented results originating from research classified as "curiosity-driven" are surely too numerous to recount. It is also far from infrequent that those conducting basic investigations are aware of the important, albeit long term, clinical applications of their "curiosity-driven" research.

The separation of basic and clinical biomedical research and the sale of research institutions that have been responsible for so much basic research that also found major biomedical applications would hinder not only new biomedical discoveries, but also the transfer of such discoveries from the laboratory to the bedside. Nevertheless, should such a "sell off" of LMB and Mill Hill be seriously contemplated, I am certain that many institutions in the United States and elsewhere will happily "bid" to attract the scientists responsible for so much of the scientific excellence in the United Kingdom. This will naturally be followed by indignant cries of "brain drain", the loudest of them from the proponents of this intellectual fire sale.

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The zootype and the phylotypic stage

J. M. W. Slack, P. W. H. Holland and C. F. Graham

What is it that defines an animal? The definition provided here, made on the basis of developmental biology, suggests methods for resolving phylogenetic problems.

WHAT is an animal? According to elementary textbooks of biology it is an organism that feeds, moves and responds to stimuli. But this definition is purely behavioural. Since the time of Geoffroy St Hilaire, there has been no morphological concept of what an animal really is. We propose that an animal is an organism that displays a particular spatial pattern of gene expression, and we define this pattern as the zootype (see Fig. 1). The zootype is expressed most clearly at a particular stage of embryonic development: the phylotypic stage for each individual taxon (Fig. 1 periphery). Hence the phylotypic stage is of critical importance not only for defining individual body plans, but also for relating these body plans across the whole animal kingdom. We believe that application of the concepts of zootype and phylotypic stage will establish a new programme of research in animal phylogeny.

The zootype

Recent work in developmental biology has shown that there is a class of gene that specifies relative position within the body. Although such genes are an essential link in the causal chain of spatial patterning, the boundaries of expression need not correspond to the boundaries of structures formed later, and the same combination of states of gene activity may lead to the formation of different structures in different organisms. Mutations within these genes, in the fruit fly *Drosophila*, transform defined regions of the body into the character of an adjacent region. Homologous genes exist in vertebrates, and where it has been possible to mutate them, shifts in morphological character have also been produced (reviewed in ref. 1).

The Hox cluster genes are the best known of these. They were originally discovered in *Drosophila*^{2,3} (Figs 2, 3), and are a subset of a wider class of genes that occur in all eukaryotes and contain a sequence motif called the homeobox (reviewed in refs 4, 5). This is a DNA-binding domain, and all homeobox-containing genes are thought to be transcription factors that control the activity of other genes. The discovery of the first Hox cluster type homeobox-containing gene outside *Drosophila*⁶ caused great excitement, and it was quickly proposed that the homeobox would become a 'Rosetta stone' for the study of animal

development, enabling us to read the epigenetic code of other animals on the basis of our understanding of *Drosophila*⁷.

By 1989 it had been found that there existed a similarity of spatial order of expression of Hox cluster homologues between embryos of *Drosophila* and mouse^{8,9}. The comparisons between insects and vertebrates have been extended and are now very well described and analysed^{1,10-12}. We know that there are four homologous clusters of Hox genes in vertebrates. Expression of the genes at the 3' end of each cluster commences at anterior body levels of the embryo, and for each gene moving along the chromosome in a 5' direction, expression commences at a more posterior level. In *Drosophila* there is a single, albeit split, gene cluster comprising the Antennapedia and Bithorax complexes, which shows clear homology to the vertebrate clusters. The relationship between gene position within the cluster and relative anterior expression limits in the embryo is the same as in vertebrates. The nomenclature of vertebrate Hox cluster genes has now been revised such that the genes within each cluster are numbered according to the position of the gene in the cluster¹³.

Although the Hox cluster genes are the best known, there are other examples of conservation of expression pattern of position-coding genes. Recently the vertebrate homologues of the *Drosophila* anterior genes *orthodenticle* and *empty spiracles* have been shown to be expressed in the head of the mouse embryo¹⁴. This extends the similarity of anteroposterior pattern into the head, often thought of as an evolutionary novelty in the vertebrates¹⁵. There is also a similarity of the *even-skipped* expression region, which lies in the extreme posterior of the mouse, *Xenopus* and grasshopper embryos¹⁶. (In *Drosophila* the expression of *even-skipped*¹⁷ is complicated by its early pair-rule function, which may have no counterpart in distantly related organisms.) On the other hand, there are many other genes that we believe to be of developmental importance and that have homologues between animal phyla, but which do not show similarity of expression patterns between *Drosophila* and mouse, for example, *engrailed*, the *Pax* genes, *Pou* genes and *Wnt* genes.

This much is familiar to developmental biologists, but even they may not realize how widely spread the comparisons now are. It has been shown that

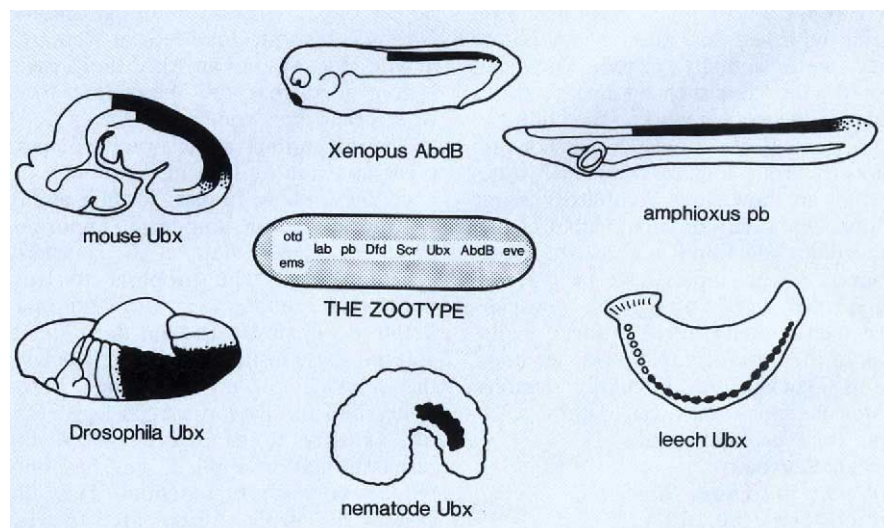


FIG. 1 The zootype (centre), showing the spatial order of anterior expression limits of the Hox cluster and some other genes. Around the zootype are shown phylotypic stages of various animals, showing the expression of individual genes as seen in whole mounts. *Drosophila* nomenclature is used for the genes: otd, *orthodenticle*; ems, *empty spiracles*; lab, *labial*; pb, *proboscipedia*; Dfd, *Deformed*; Scr, *Sex combs reduced*; Ubx, *Antennapedia-Ultrabithorax-abdominal A group*; AbdB, *Abdominal B*; eve, *even-skipped*. The specific nomenclature for animals and genes shown is as follows. Mouse, Hox 1.1 (=Hox A7); *Xenopus*, XlHbox 6; amphioxus, Amphiox 3; *Drosophila*, Ubx; *Caenorhabditis elegans*, mab-5; *Helobdella*, Lox-2.



FIG. 2 Ubx protein visualized in the retracted germband stage of a *Drosophila* embryo by immunoperoxidase staining. Lower, ventral surface view; upper, optical section through deeper level. Anterior is to the left. Photographs courtesy of P. Ingham, ICRF Developmental Biology Unit.

genes of the Hox cluster have comparable expression patterns in amphioxus¹⁸, leech^{19,20} and nematode^{21,22}. Each of these carries a special significance. The amphioxus-vertebrate comparison suggests that the vertebrate head is homologous to the anterior, but not cephalized, segments of the lower chordate. The leech-insect comparison shows that the patterns persist even though the modes of segment formation are very different. The nematode results show that the same expression pattern can be present in an organism with an invariant cell lineage and a very small total cell number. In other words, the Hox cluster genes really do seem to encode relative position within the organism rather than any specific structure, and the patterns are conserved despite major shifts in other developmental mechanisms. Some people have proposed that Hox gene activities, or combinations of activities, do encode particular structures, for example the different types of vertebrae in mammals²³. It is likely that these associations will be found within a phylum but the inter-phylectic comparisons suggest that the basic function is to code for relative position. The existence of such 'positional values' was predicted some time ago by Wolpert on the basis of classical experimental embryology²⁴. He made the explicit prediction that the coding system should be universal, and this does now seem to have been borne out.

Hox cluster genes are also present in *Hydra* (phylum Cnidaria). Although the normal expression pattern here is not known, when the head is amputated and a new head regenerates from the stump, the labial homologue, *cnos-1*, comes on

before the *Dfd* homologue, *cnos-2* (ref. 25). In organisms capable of bipolar regeneration, the rule is that the extreme positional coding becomes established first and intermediate codings are then intercalated from anterior to posterior²⁶. So if *cnos-1* is turned on before *cnos-2* in regeneration, this suggests that *cnos-1* represents a more anterior coding than *cnos-2*, consistent with the expression of their homologues in other animals. The presence of Hox cluster genes has also been reported in the fluke *Schistosoma* (phylum Platyhelminthes), although no expression data is yet available²⁷.

Because it looks strongly as though a system of gene expression patterns, comprising the Hox cluster type genes and some others, do encode relative position in all animals, it is probable that this system is very ancient and was in place in the common ancestor of all modern animals (Fig. 4). We now suggest that this character should be adopted as the defining character, or synapomorphy, of the kingdom Animalia. We propose that it be called the zootype.

The phylotypic stage

It is widely appreciated that each animal phylum can be represented by a particular general body plan²⁸. It is less widely known that there is a stage of development at which the precursor to this body plan is actually visible. This was first defined as the stage of the *Körpergrundgestalt* by Seidel²⁹, and called the phylotypic stage by Sander³⁰. The phylotypic stage can be variously defined as the stage of development at which all major body parts are represented in their final positions as undifferentiated cell condensations, or the stage after the completion of the principal morphogenetic tissue movements, or the stage at which all members of the phylum show the maximum degree of similarity. The following seem to meet these criteria: the tailbud stage for the vertebrates; the fully segmented germband stage for insects; the fully segmented, ventrally closed stage for leeches; or the nematode after the completion of most embryonic cell divisions.

The phylotypic stage of development is not the earliest stage. Early stages can be quite variable even within phyla or classes. Some examples of such divergence are the arrangements of extraembryonic

membranes in different groups of mammals³¹, the modes of segmentation in short and long germ insects³², or the existence of holoblastic and meroblastic cleavage in different groups of fish³³. It is generally presumed that this variability of early stages results from adaptation to particular types of reproductive strategy or to the demands of embryonic nutrition. The conservative phylotypic stage lies between the variable early stage and the variable late stage at which the morphology of the postembryonic organism is formed in miniature, and is again susceptible to adaptive change.

It is a remarkable fact that, for each individual phylum so far examined, the embryonic stage at which the zootype is most clearly displayed is the phylotypic stage of the group concerned (see Fig. 1). The genes of the zootype are not, in general, activated in the earliest stages of development, and although expression may persist for some considerable time, the peak expression, and the simplest pattern of expression, is displayed at the phylotypic stage. This association gives us confidence that the independent proposals of evolutionary conservatism of the zootype and of the phylotypic stages are indeed well founded.

Implications of the zootype

From the existing data it seems that non-animal multicellular eukaryotes such as plants, fungi and slime moulds have homeobox-containing genes, but that these do not fall into the homologous families of the genes whose expression patterns comprise the zootype (T. R. Burglin, personal communication). There are also no similarities of expression between these organisms and animals. The definition of the zootype therefore gives us, for the first time this century, a morphological criterion for what an animal is. It is more precise than the behavioural criteria used hitherto, and has both theoretical and practical implications.

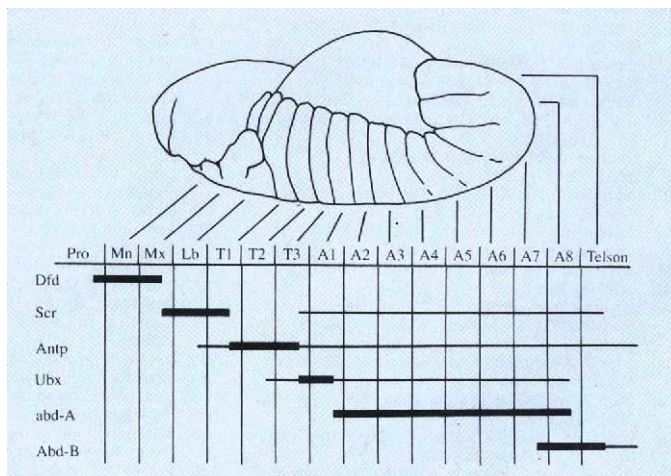


FIG. 3 Expression domains of Hox cluster genes in the *Drosophila* embryo.

First, we may consider the reason for the persistence of the zootype. The evidence from the effects of overexpression, or mutation to inactivity, of the genes of the zootype, is that their function is to encode different relative positions within the embryo. Their functions are informational rather than executive. Information units are arbitrary in that one could be substituted for another without affecting function, so it seems probable that the persistence of the zootype indicates common ancestry rather than convergent evolution driven by considerations of molecular efficiency. So we believe that there was once a primordial multicellular ancestor of all existing animals and this ancestor was the first organism to possess the zootype (Fig. 4). The zootype has persisted ever since, perhaps because, although it is arbitrary, mutational change to it would be too disruptive to the rest of the developmental programme of a plausible animal. We are unable to say anything about the visible morphology of the proto-animal because the zootype is a system of positional information and does not necessarily encode any particular structures.

Second, although the zootype is not visible on casual inspection, the presence of the genes can be established using the polymerase chain reaction on preserved specimens and, so long as a phylotypic stage is available, the expression patterns can be visualized on preserved specimens by *in situ* hybridization. It is therefore a practical taxonomic tool for deciding the affinities of various 'borderline' organisms. These would include the sponges (phylum Porifera), the Mesozoa, the Placozoa and various groups of Protozoa³⁴.

Third, although the definition of phy-

lotypic stage given above is clear enough for most groups, there are ambiguities. For example, most echinoderms have motile larvae. These come with various morphologies (pluteus, auricularia, vitellaria and so on) which do not entirely correspond to the class divisions defined by adult morphology³⁵. Is the phylotypic stage the larva or the metamorphosing adult? If we accept the evidence from the other phyla then the zootype must be displayed at the phylotypic stage, but need not be at other stages. Hence examination of the zootype in echinoderms could tell us which stage is really phylotypic. This would tell us which stage should really be compared to other phyla in the establishment of super-phyletic groups such as the Deuterostomia. Evolutionary biologists may also be able to decide whether those echinoderms without larvae³⁶ are primitive or show secondary loss of the larva.

Could parts of the zootype itself be subject to secondary loss? The zootype has clearly persisted for a very long time in evolutionary history and this may suggest that the maintenance of a complex morphology needs the system of positional information which is provided by the zootype. However, it may be possible for highly degenerate creatures, such as parasites with little remaining morphology, to have lost parts of the zootype. To a strict cladist this possibility would mean that the zootype was not a good synapomorphy for Animalia. We

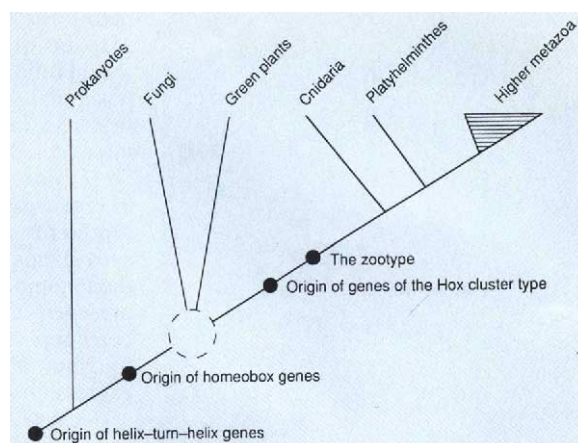


FIG. 4 Origin of the zootype on the evolutionary tree. The Hox cluster genes are a subset of the homeobox genes, which are in turn a subset of genes encoding DNA-binding proteins of the helix-turn-helix class.

would take the view that a plant which has lost its chloroplasts remains a plant because of the weight of other correlated characters, so perhaps the same attitude should be taken to the possibility of secondary loss of parts of the zootype.

Finally, the recognition of the zootype could provide a rational basis for large-scale animal taxonomy. The existing groupings above the level of the phylum are somewhat insecure, mainly because of the difficulty of attempting to find morphological homologies between different body plans³⁵. But if the zootype is common to all animals, then the next layer of genes controlled by the zootype codings is likely to represent the next fundamental level of body patterning. Once these genes have been identified and their functions understood, then they will make up the basic characters for defining phyla and super-phyla. This area of molecular developmental biology must now be rated as a particularly important one for those studying taxonomy.

To an extent the zootype represents a rehabilitation of the concept of the archetype of all animals, promoted by Geoffroy St Hilaire in the first part of the nineteenth century³⁷. Little could he have known that the zootype can best be observed at a stage of development that was just beginning to be described in those days, and that its observation would require techniques that would not be invented for nearly 200 years. But the zootype is not just a novel means of defining the animal kingdom; it also leads us to make a number of testable predictions about phylogenetic problems. We await the results of these tests with interest. □

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Can quantum theory be understood?

Physics has a more urgent responsibility than it appreciates to make the most general tool of its trade more widely understood, to which end proofs positive are more relevant than abstractions.

SOME years ago, a group of able physics teachers met to plan a new curriculum for the sixth forms of British schools and were asked, "What will you put in about quantum mechanics?" To a man and woman they shook their heads, saying that they did not know, but that they were prepared to follow whatever instructions they were given. In the event, the curriculum turned out to be more adventurous than many feared, but the willingness of normally determined people to follow guidance about quantum mechanics is a telling proof of how poorly the matter is generally understood.

So much is easily verified by asking artlessly at dinner parties and the like some variant of the question "What does quantum mechanics mean to you?" With luck, the response may be the opinion that quantum theory implies that nothing is as certain as Newton said or, with some brave extrapolation, that nothing at all is certain.

A modicum of technical education, on the other hand, usually yields one or both of two elaborations of this doctrine. One is a reference to the Uncertainty Principle and to the limitations that it imposes on the accuracy of physical measurements. Another is the doctrine that "energy is quantized", offered as an explanation why the electronic states of atoms and other structures, such as metals, can accommodate electrons only in states whose energy is predetermined and, often, precalculable.

The trouble with even these statements, their imprecision apart, is that they are too abstract to carry general conviction. Worse, they seem logically unconnected with each other. Why, for example, should the Uncertainty Principle, which would seem to make quantities fuzzy, be linked with the apparently contradictory notion that the energy of an electron in, say, the ground state of a hydrogen atom is exquisitely exact? Is quantum mechanics about uncertainty or the opposite?

It hardly helps the general understanding to remark that the frequency span of a spectral line is partly a measure of the lifetime of the more energetic state involved, and that time and energy are linked as if they were any other pair of conjugate variables in classical mechanics. What on Earth, the question will be, are they? It will seem irrelevant that the concept, due to William Hamilton, long antedates Einstein and Bohr.

What quantum mechanics, and perhaps the reputation the rest of physics, needs is a series of phenomena that will stick in the

general mind and serve as repeated reminders of what the field has to say for itself. That, after all, is why biology enjoys such a good press. From infection to digestion to reproduction, everybody has in mind first-hand experience of body as machine. Physics, and the quantum mechanics part of it especially, is necessarily more artificial, requiring experiments of some kind. But that impediment is not insurmountable.

There is, for example, the question of the production of electron-pairs. The world is full of photographs (some going back to before the discovery of the positron) taken in cloud chambers and other devices placed in magnetic fields that display the elegantly backward curving tracks of an electron and a positron apparently diverging from a point in space on which nothing else impinges. The standard tale is that there is instead a photon, invisible because it has no electric charge, which has created an electron pair from nothing. The response invited is something along the lines of "Isn't that astonishing!" The impression is reinforced that physics is either akin to magic or that it seeks to be.

Why not tell it like it is? The Uncertainty Principle is a good starting point. That empty space in which the electron-pair appears cannot be exactly empty, right, because no entity can be as exactly defined? And in reality, any patch of vacuum is potentially a source of any substantial particle you may specify (except, it seems, the 17-keV neutrino and, for the time being, the *top* quark)?

So what more natural than that a photon traversing such a patch of space, especially one disturbed by the presence of an atomic nucleus (necessary on kinematic grounds) should exchange its energy for that of an electron-pair? The crucial element in this pedagogical exercise is to plead that the Uncertainty Principle ensures that there is qualitative as well as quantitative uncertainty, but that is simply to tell it like it is.

Sadly, when physics is taught to people not intending to be physicists, the moral usually drawn from the picture of the electron-pair is more often that it illustrates again Einstein's old saw that $E = mc^2$, which is proper but unadventurous. Why not use the electron pair as a text for a sermon about the fluctuations of the vacuum, the idea that nothing is potentially the source of everything? That might stick in the general mind.

Casual flicking through physics journals will then provide endless ammunition. If the vacuum is a potential source of electron-

pairs, so too is it potentially a source of photons. The frequency of the radiation emitted by a circulating beam of electrons in a synchrotron can be calculated from the work of Maxwell and Lorentz, but the energetic photons that biologists use for X-ray studies of protein crystals have been physically conjured out of the vacuum by nothing more substantial than the curvature of the electric current represented by the circulating electrons (which in this case are not destroyed).

Here, for what it is worth, is the neatest experiment yet carried out to demonstrate that the vacuum is potentially an endless source of photons, even when their effects are never manifested. A group at Yale University (Sukenic, C.I., *et al. Phys. Rev. Lett.* **70**, 560; 1 February 1993) has constructed a cavity consisting of the wedge-shaped region between two gold-covered surfaces flat to within 3 nanometres. The opening of the wedge is determined by sliding a nickel foil 1.2 μm thick between the flats.

In such a cavity, only some frequencies of the radiation field can be supported, as they say in the computer business. So what happens if you shoot a beam of ordinary unexcited sodium atoms through the wedge-shaped space? Because the wedge does not allow many of the radiation frequencies found in larger patches of vacuum, the energy of the electrons in the sodium is shifted from what it would ordinarily be. That, at least, is the prediction. What matters is that it appears to have been confirmed by a measurement of the transmission of a carefully tuned laser beam through the emergent beam of sodium atoms. The experiment has not merely demonstrated that the vacuum is not devoid of substance, but that the consequences are apparent even when there is no magical appearance of something unexpected.

To be fair to Sukenic and his colleagues, they are not simply concerned to show that quantum mechanics has substance, but to demonstrate the reality of what is called the Casimir-Polder force the attractive force between two nearby conducting plates occasioned simply by the absence in the intervening cavity of radiation frequencies that cannot, because of the geometry, exist. The force has previously been measured directly, as has been the increased lifetime of an excited atom trapped in such a space. These phenomena, which are artificial, may be a long way from everyday experience, but they are proof positive, not abstractions.

John Maddox

The fruits of cooperation

Ann Hill and Helen Chapel

THREE weeks ago, *Nature* published an account of the identification of the gene responsible for the human primary immunodeficiency disease, X-linked agammaglobulinaemia (XLA)¹. By careful genetic mapping of a disease locus, Vetrie and coworkers discovered a previously unknown protein-tyrosine kinase gene involved in B-cell development.

Now, five papers have popped up almost simultaneously describing how the opposite pathway has led to description of the cause of another X-linked immunodeficiency disease; two of the reports appear in this issue (pages 539² and 541³), the others in *Science*⁴, *Cell*⁵ and *Proceedings of the National Academy of Sciences*⁶. The disorder is X-linked immunodeficiency with hyper-IgM (HIGM1), a disease characterized by failure of T-B cell cooperation. It was knowledge of the essential part in the T-B cell cooperation played by the newly discovered CD40 ligand that resulted in identification of this gene as the culprit.

The interdependence of T and B cells has long been recognized, giving rise to the designation of a subclass of T cells as 'helpers' because of their obligatory role in enabling B cells to produce antibodies of different isotypes and to establish immunological memory. Both soluble factors (cytokines) and cell-cell contact are required for effective help. Cell-cell contact achieves the critical cross-linking of the B-cell activation marker, CD40. This enables B cells to proliferate and, in the presence of appropriate cytokines, to 'switch' the isotype of immunoglobulin they produce from IgM to IgG, IgA or IgE. CD40, apparently a member of a growing family of receptors related to the tumour necrosis factor (TNF) receptors⁷, was until recently a receptor without a known ligand. Then, within the past six months, three groups reported the cloning of its murine and human ligands⁸⁻¹¹. The CD40 ligand, it turned out, is a homologue of TNF- α and is expressed on the surface membrane of activated CD4⁺ (helper T cells) as well as on some CD8⁺ T cells.

A crucial lead in the story told in the new papers was the mapping of the gene for the CD40 ligand to the long arm of the X chromosome (Xq26.3-27.1)^{4,9}. Also known to map in this region (Xq24-27) was the unidentified gene responsible for HIGM1^{12,13}. This is a rare primary immunodeficiency syndrome characterized by the inability of B cells to undergo isotype switching: B cells produce normal amounts of IgM appropriately in response to antigenic

challenge, but fail to switch to produce IgG, IgA and IgE and to establish memory. T-cell dysfunction was suspected in the disease because, unlike other antibody-deficient patients, some HIGM1 patients are surprisingly prone to certain infections commonly associated with T-cell deficiency, such as *Pneumocystis carinii* pneumonia and cryptosporidial diarrhoea. T cells were further implicated by an elegant study which demonstrated that HIGM1 B cells could be made to produce normal amounts of IgG, IgA and IgE if co-cultured with a malignant human T-cell line¹⁴.

With this background, the five groups²⁻⁶ set out to investigate the role of the CD40 ligand in HIGM1. They looked at a total of 18 unrelated individuals with HIGM1. Activated T cells from 17 of these individuals failed to bind an engineered soluble form of CD40; in the eighteenth, only weak binding was seen⁴. The CD40 ligand genes from 13 patients were then sequenced and in 12 a different point mutation or deletion was discovered. DiSanto *et al.*³ show that, in three of their cases, the defective gene is carried by the asymptomatic mother. In a fourth case the defect appeared to be a new mutation. Four of the groups provide experimental evidence that the functional defect in B cells seen in HIGM1 is indeed due to failure to cross-link CD40. Cross-linking of CD40 restores the ability of HIGM1 B cells to proliferate and to undergo isotype switching when co-cultured with appropriate cytokines^{2,4-6}.

As icing on the cake, three of the groups describe experiments demonstrating that the defective CD40 ligand gene is indeed responsible for the T cells' inability to bind CD40. DiSanto *et al.*³ take advantage of a polymorphic microsatellite repeat in the 3' untranslated region of the CD40 ligand that they have identified. The mother of one patient carried two allelic forms of this marker. Because X-chromosome inactivation is random in the HIGM1 carrier, only half of her activated T cells express functional CD40 ligand: all of the T cells that failed to bind soluble CD40 displayed the microsatellite marker associated with the defective CD40 ligand gene. Allen *et al.*⁴ have transfected cell lines with wild-type CD40 ligand genes or with identified HIGM1 mutant alleles. Whereas the wild-type transfectants bound CD40 and induced isotype switching in normal B cells, the lines transfected with the mutants failed to do so. Aruffo *et al.*⁵ report similar results with soluble forms of the mutant CD40 ligand.

So knowledge of the pivotal importance of the CD40-CD40 ligand interaction in B-cell differentiation and isotype switching has led rapidly to identification of the CD40 ligand gene as responsible for HIGM1. A closer study of the phenotype of the disease now prompts further questions about the function *in vivo* of this ligand-receptor pair. There are two characteristic features of HIGM1¹³ which to our minds are not easily explained by the currently identified role for the CD40 ligand in T-B cell cooperation. One is the mechanism by which inadequate T-cell function fails to control intracellular parasites such as *Pneumocystis carinii*; further study of HIGM1 T cells may help to shed light on this. It may be relevant that CD40 is expressed on epithelium, interdigitating cells and basophils as well as on B cells; to date, the CD40 ligand has been identified only on T cells. The other, even more puzzling, feature is the intermittent drop in granulocyte numbers which affects about half of the sufferers from this disease. Is the CD40 ligand involved in this in some way or is this really an autoantibody problem?

For HIGM1 patients and clinicians, carrier detection will be an immediate benefit. Specific therapies, with genes or recombinant proteins, are now possible, although most patients remain well with adequate antibody replacement. The benefits of human gene therapy and the outcome of animal trials with the recombinant proteins are eagerly awaited. For scientists, the heterogeneity of primary immunoglobulin deficiencies⁵, in both phenotype and inheritance patterns, provides natural opportunities for identifying genes involved in the basic biology of antibody production. □

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A window to the core

Michael E. Wyssession

THE Lop Nor nuclear test last year in China, the largest for more than ten years, gave seismologists an unusually well-defined source of seismic waves with which to study the Earth's interior. Vidale and Benz, whose results appear on page 529 of this issue¹, used these to find a distinct island of material at the boundary between core and mantle, nearly 3,000 kilometres down. This is smaller than anything seen before at such depths, and is a feature not expected from the results of previous studies.

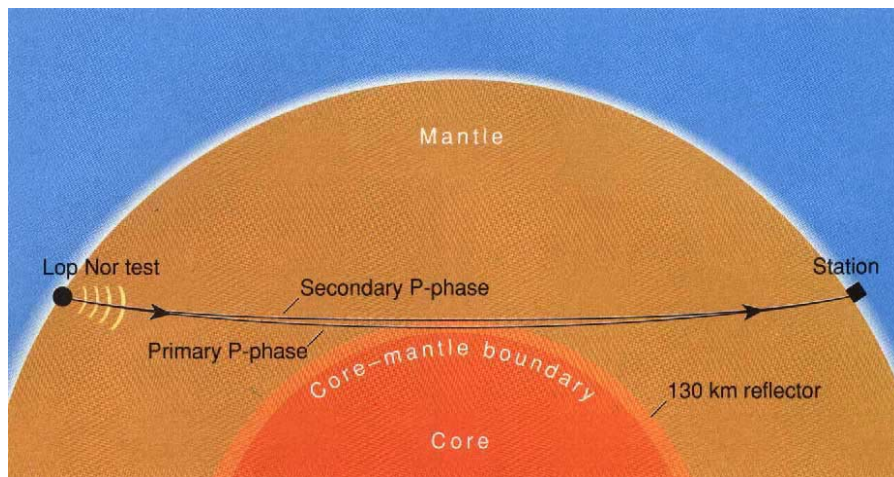
The bottom 200–300 kilometres of the mantle, known as D'', is seismically distinct from the rest; its thickness and make-up seem to vary laterally on a scale comparable to the continents of the lithosphere, the Earth's top 100 kilometres (ref. 2). At great depths, though, horizontal resolution is poor, and Vidale and Benz's discovery of an anomalous layer of seismically fast material 130 km thick and about 300 km wide is a considerable step forward. This width is an order of magnitude smaller than any other feature yet identified at the boundary between the core and the mantle, and the depth suggests that D'' (which is about 250–300 km thick here³), must contain multiple layers.

Organization

This improvement in resolution stems from an organizational rather than a technological breakthrough. By piecing together records from 14 US and Canadian regional networks of short-period vertical-component seismometers, the authors assembled an array of more than a thousand records of teleseismic compressional (P) waves from the Lop Nor test on 21 May 1992. Most teleseismic studies have to use few stations and many earthquakes, the places and times of which cannot be pinned down nearly so accurately. The seismometers used here cannot be used for teleseismic shear (S) waves or long-period P waves, but they give high resolution for the P velocity structure, and are well suited for sampling nuclear explosions, which create much high-frequency compressional seismic energy.

By analysing the best 477 records of the 1,062 available from the Lop Nor test, Vidale and Benz identified waves arriving 1–3 seconds after the direct P phases, and interpreted them as wide-angle reflections off a seismically fast layer within D'', roughly beneath Alaska. Such secondary arrivals arise when the fast layer bends rays entering it, so that more than one arrival from the same

phase reaches a given station (see figure). The secondary arrivals were seen from a patch of the boundary roughly 300 km across, but the clarity of the signals suggests that the region is slightly larger — only slightly, because it cannot be seen in nearby regions of D'' sampled



Vidale and Benz examine seismic waves that interact with a 130-km-thick seismically fast layer at the base of the mantle in such a way that waves following different paths can arrive at a single location. In this process, called triplication, the waves arrive a few seconds apart, with the primary P arrival travelling deeper through the fast layer and the secondary P phase just skimming the top of it.

by waves from the Lop Nor test and from earthquakes.

The thickness and percentage increase in velocity in the fast layer produce similar effects on the signal, but the clearness of the arrivals helps constrain the anomaly to being about 130 km thick with a P velocity 1.5 per cent faster. No sign of this appeared in the authors' earlier investigation (using earthquakes) of a 350-km patch of D'' just to the south⁴. There, using hundreds of ScP and PcP core reflections, they found no observable layering, topography of less than 500 m, and a very sharp transition from mantle to core in less than a kilometre.

The results are all the more intriguing because they do not occur with previous studies. Fast D'' layers a few hundred kilometres thick have shown up in short-period P waves in other parts of the world, but not beneath Alaska⁵. When, however, we look at intermediate shear-wave ScS precursors (shear energy attenuates too quickly in the mantle for reliable short-period studies), a fast layer does show up. In these results it appears to be 200–300 km thick and 2.75 per cent faster than expected³. To complicate matters further, studies of the average D'' structure at large scales (using both whole-mantle tomography and core-diffracted waves) have consistently

shown faster-than-average S velocities^{6–8} but slower-than-average P velocities beneath Alaska^{8–10}. We are beginning to see the effects of different scales of lateral heterogeneity in D'', much the way that crustal seismic velocities vary at scales of mountain belts and continents. For example, work on ScS waves bounced off the core has identified alternating fast and slow velocity anomalies at the base of the mantle with wavelengths ranging from 500 to several

thousand kilometres, although the resolution is too poor to see finer structures^{11,12}. It seems that Vidale and Benz have located a small regionally fast layer within a larger area of generally slow P velocities.

The fact that we actually find any seismically fast regions at the base of the mantle is surprising on thermodynamic grounds. D'' is thought to be a thermal boundary layer with a temperature increase of 1,000 °C or more across it, and this should cause a decrease in seismic velocities. Indeed, D'' has a nearly flat velocity gradient with depth, whereas the seismic velocity of the rest of the lower mantle increases with depth along its thermodynamic adiabat.

To explain the observations, we may have to call on analogies with surface processes. Just as much lateral heterogeneity at the Earth's surface is the result of lateral plate motions, so D'' might be shaped by active lateral convective sweeping. Material leaving the Earth's surface as subducting slabs is recognized to cause seismically fast anomalies; seismically slow anomalies at the base of the mantle may show where hotspot mantle plumes originate.

Liquid iron reacts with silicate perovskite, the primary component of the lower mantle, to form a suite of by-products. Vidale and Benz suggest that

a 25 per cent increase in one of these, stishovite, could explain their observations. A locally high concentration of stishovite, which apparently changes phase at pressures corresponding to D'', would account for the increase in P velocity, and might also be less visible to S waves, thus explaining how it was missed in other studies. This, though, is speculation — a similar physical effect could have come from a phase change in perovskite or magnesiowüstite (the other main lower-mantle component), from anisotropy in perovskite (where waves travelling through the same material in different directions travel at different speeds) or from a purely thermal anomaly caused by the sinking of cold, dense lower-mantle dregs.

Heterogeneity

The increased resolution of this study has not only identified a new mantle layer in a region where one has not been seen before, but it has also shown that we will have to make the next conceptual step in attempting to visualize this layer. Geoscientists have become comfortable with the idea that the layer we call D'' varies in thickness and composition, but we may have to stop thinking of it as one homogeneous layer. To depict the Earth's lithosphere as one layer would eliminate all the subtleties of its crust, and we should expect D'' to be just as complex. For a start, chemical and thermal boundary layers, each with their own seismic signature², may be superimposed. At a more elaborate level, lateral variations in composition and temperature probably combine with effects such as mineralogical phase changes, anisotropy and topography of both the top and bottom of D''. Such a complicated situation would be hard to picture if it were not the same way with the Earth's other main boundary layer, the lithosphere. □

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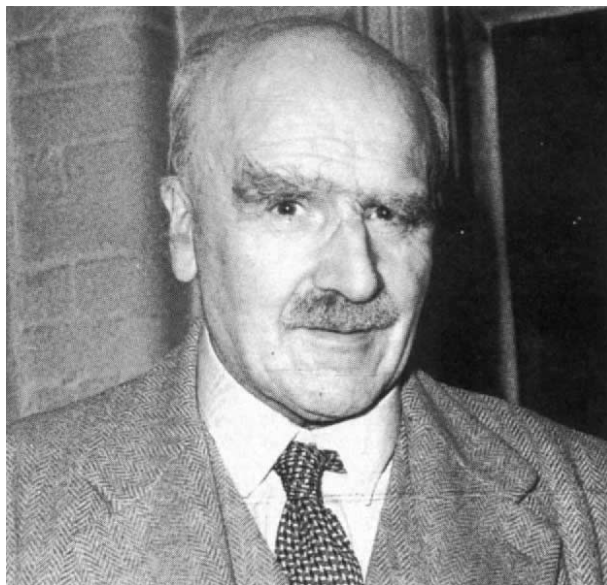
The genetics of speciation

John Brookfield

ON page 532 of this issue¹, H. Allen Orr tackles a long-standing issue for evolutionary biologists, the genetic basis of Haldane's rule. In a cross between two species, the offspring of one sex are often inviable or sterile, and Haldane's rule has it that the heterogametic sex (that with two different sex chromosomes, X and Y) is the one affected². Orr now finds that the basis of this phenomenon is not as straightforward as has been thought — in short, he shows that the genetic causes of the rule differ

duction of F₁ hybrid females that have both their X chromosomes from one parental species. Such females are fully fertile, despite the absence of X-chromosomal material from one parent, and homozygosity for X-linked mutations from the other⁴. Now, however, Orr shows that the opposite result is found if F₁ viability is examined rather than fertility. He describes two interspecific crosses in *Drosophila*, between *D. simulans* females and *D. teissieri* males, and between *D. melanogaster* females and *D. simulans* males, in which Haldane's rule operates for viability, with viable F₁ females and inviable males. In these crosses, female viability is abolished if both X chromosomes come from the female parent.

These results represent further successes in the understanding of speciation which stem from a focused experimental approach in which realistic genetic experiments are performed in well-characterized organisms. Progress in the genetics of reproductive isolation has been possible through the tracing



J. B. S. Haldane — his rule is OK, but what is its genetic basis?

of chromosomes through interspecific crosses of *Drosophila* using visible mutations. Indeed, Coyne and Orr⁵ have demonstrated that two general rules of reproductive isolation hold. The first is Haldane's rule. The second is that the chromosome contributing most to reproductive isolation is the X chromosome; this is true even when it is a reduction in F₁ female fertility that is mapped.

How can Haldane's rule be explained if the ideas of balance and of the recessivity of fertility mutations are incorrect? And what is the explanation of the preponderance of X-chromosomal effects? One helpful model⁶ postulates that hybrid male sterility is the result of mutations affecting male fertility on the X chromosome. The model assumes that the mutations are usually recessive and thus accumulate preferentially on the X by hemizygous selection in males. Their recessivity is clearly essential to the model but hard to explain on *a priori* grounds. These mutations are advantageous in the context of their own species, but very harmful when combined with auto-

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GLOBAL WARMING

somes from another species. So the reason for Haldane's rule is that the mutational changes between the species predominantly affect fertility in males. Female hybrids are therefore fertile not because female fertility mutations are recessive, but because they do not exist. With increasing evolutionary separation it is found that fertility in both sexes is lowered in hybrids. The genes causing the reduction in female sterility also map preferentially to the X. This must be explained by selection in the males for mutations affecting fertility in both sexes. But because mutations affecting both male and female fertility are rare, female sterility mutations on the X will be rarer than male fertility mutations, and hence there will be a range of between-species divergences for which Haldane's rule will hold.

What, in this model, is the significance of the new results for F_1 viability? Unlike mutations affecting male fertility, those affecting male viability, selected in hemizygous males as a result of their recessive advantageous effects, are likely also to have effects in the female. So species will differ by mutations affecting both male and female viability. The female hybrids, however, will not show the harmful effects of these mutations, because the mutations are on balanced X chromosomes and are recessive. Creating hybrids with both Xs from one species uncovers the harmful effects of these X-linked inviability mutations. For these mutations, the traditional explanation of Haldane's rule is correct, thereby tempting us to question the generality of the result that female fertility is high in attached-X crosses. In other species pairs, also showing Haldane's rule for fertility, female fertility genes could have diverged but this effect have been hidden by their recessivity.

The most promising aspect of this approach, of course, is that the power of *Drosophila* genetics and molecular biology is such that genes that can be mapped can ultimately be investigated at the molecular level. This will show whether 'speciation genes' are a specific class of genetic entities, or whether the generation of reproductive isolation is merely an inevitable by-product of 'normal' evolutionary changes in genes affecting viability and fertility. □

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Carbon reserves released?

Robert S. Webb and Jonathan T. Overpeck

THE Earth's capacity to store carbon, in vegetation and soil, could have either of two opposed effects on the global response to man-made greenhouse gases: global change could be reduced if carbon compounds are taken up, or amplified if the reservoirs give up some of their contents. In the long term, it seems^{1,2}, terrestrial carbon storage will remove carbon from the atmosphere. But on pages 520 and 523 of this issue^{3,4}, Oechel *et al.* and Smith and Shugart show that the carbon stores may add

close to the lower limit of the range of values (0.2–0.7 Gt C yr⁻¹) that Tans *et al.* sought. The authors suggest that the observed net production of carbon may be the new status quo for high-latitude ecosystems as they respond to greenhouse warming.

Equilibrium studies^{1,2} of climate change have suggested that in most cases the input of carbon by high-latitude ecosystems will be more than compensated for by sequestration in middle- and low-latitude ecosystems. Smith and Shu-

IMAGE
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Global warming is changing the balance between photosynthesis and microbial decomposition in the Alaskan tundra: a carbon sink becomes a carbon source.

to the CO₂ burden of the atmosphere over short durations. Predictions of the increasing atmospheric carbon load over the next 50–100 years may have to be modified to take this amplification into account⁵.

Since the last ice age, high-latitude land areas have been a sink for CO₂, with long-term organic-carbon accumulation through photosynthesis exceeding microbial decomposition^{6,7}. But Tans *et al.*⁸ have argued that there must be a present-day high-latitude CO₂ source to balance a north–south gradient observed in atmospheric CO₂. Oechel *et al.*³ have measured the CO₂ flux over the Alaskan tundra for five summer seasons, and suggest that they have found the first conclusive evidence for at least one source of high-latitude CO₂. They postulate that an indirect effect of the observed global warming, and local warming in the north slope of Alaska, may have switched the tundra from net carbon storage to production. Extrapolating these observations to the whole high-latitude belt, Oechel *et al.* calculate that an additional 0.19 gigatonnes of carbon were injected into the atmosphere this way in 1990. This estimate is

gart's analysis⁴ of the global transient response of terrestrial carbon storage to future climate suggests that a short-term net global loss to the atmosphere is equally plausible. They have looked at the consequences climate change will have on vegetation, allowing for different response times for vegetation mortality, successional replacement, immigration, loss and gain of soil carbon. Forcing the model with temperature and precipitation anomalies from two global climate models, Smith and Shugart simulated a new flux of carbon from terrestrial storage to the atmosphere. The transfer of carbon may approach 200 Gt within the next 100 years, amounting to a 20 per cent amplification of the projected⁹ anthropogenic input.

Although intriguing and significant, the results of the new studies should be viewed with caution. They are better than previous efforts, having used actual observations of soil carbon fluxes and simple transient simulations of global ecosystems, but they are preliminary, relying on empirical approaches to estimate changes in the global carbon budget. Certainly, the flux measurements by Oechel *et al.* suggest that the

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transient response approach incorporated by Smith and Shugart is reasonable, at least for tundra. But the simple extrapolation of the measured fluxes to other high-latitude ecosystems overlooks any regional variability in climate change, and fails to account for possible effects of increased precipitation on soil carbon storage. Clearly this kind of field work will need to be done elsewhere, for longer periods, and with careful comparisons with temperature and precipitation records.

Smith and Shugart's model omits any direct 'fertilization' effect that elevated CO₂ may have on vegetation. Nor does it include any individualistic responses of plants that could lead to the development of vegetation assemblages distinctly different from those of today. And what of the effects human activity may have on ecosystems?

Numerical models of terrestrial carbon storage will inevitably remain simplistic over the next decade. Clearly, the best way to refine them will be to see how the modelled changes reflect the real ones. It will also be worth looking to the past. Changes over the past hundred years will probably be too small to be useful, and human activity will confuse matters. But the geological record, which includes information on vegetation and soil as well as CO₂, will be well worth reproducing as a valuable test of different modelling approaches.

As the studies in this issue underline, there are uncertainties and feedback mechanisms that must be considered when trying to predict climate change. The terrestrial effects are important. But even more so is the potential for the ocean to take up carbon; current estimates^{7,10} are that an additional 1–2.1 Gt may be taken up. And as modellers come to grips with equilibrium changes, the emphasis must shift to transient processes. □

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Noggin the dorsalizer

J. M. W. Slack and D. Tannahill

OUTSIDERS must be puzzled by the fact that so many papers on *Xenopus* development open with a reference to the classical organizer graft¹, which produces a double embryo following transplantation of a dorsal lip into a host gastrula. Surely this problem must have been solved by now? How can the organizer do so many things? What is the organizer anyway? The secret of understanding early *Xenopus* development is to appreciate that there is a whole series of inductive interactions responsible for forming the body plan and that the dorsal blastopore lip of the gastrula, otherwise called the organizer, is the source region for at least three of them: dorsalization of the mesoderm, anterior–posterior patterning of the mesoderm, and neural induction. A paper by Smith *et al.* on page 523 of this issue² brings us quite near to establishing the molecular basis for the first of these activities: the capacity to dorsalize the mesoderm.

Before the mesoderm can be subdivided it must first be formed. It is generally thought that the mesoderm is induced from the equatorial region of the embryo (the marginal zone: MZ), in response to signals from the vegetal hemisphere^{3–5}, during the blastula stage of development⁶. Initially, the mesoderm exists as two territories, the ventral region occupying about 300° of circumference centred on the ventral midline, and the organizer occupying about 60° centred on the dorsal midline. This probably reflects the action of qualitatively different inducing signals from the vegetal hemisphere, and although the details remain to be resolved, there is now good evidence that both activin⁷ and fibroblast growth factor⁸ are involved in mesoderm induction. The further patterning of the mesoderm occurs during gastrulation, and takes place both in the anteroposterior axis and in the dorsoventral axis. The anteroposterior axis specification can be revealed indirectly by acquisition of regionally specific neural inducing activity^{9,10} but is not considered here.

The dorsoventral patterning of the mesoderm, normally called 'dorsalization', controls the tissue type formed: notochord from the organizer itself, then somite, kidney, lateral plate and blood islands with increasing distance from the dorsal midline. In the famous organizer graft, the grafted dorsal lip induces a second set of mesodermal territories around itself with opposite polarity to the host axis; hence the whole forms a double-dorsal duplication. Although textbook accounts of the organizer, as

indeed Spemann himself, usually emphasize its ability to induce a second neural plate from the ventral ectoderm, its capacity to dorsalize the mesoderm is just as important in the formation of the double embryo.

The dorsalizing signal is independent not only of mesoderm induction, but also of neural induction^{11–14}. Explants from dorsal and ventral marginal zones (DMZ + VMZ) of *Xenopus* gastrulae can be combined, and show that the VMZ, instead of forming blood and mesenchyme, will now form large muscle blocks and pronephric tubules. Unlike mesoderm induction, which declines sharply after the start of gastrulation, dorsalization can operate throughout gastrulation. But like mesoderm induction, the dorsalization signal is carried by a diffusible substance emitted by the DMZ. Candidate substances for the dorsalizing morphogen should be able to induce muscle from isolated gastrula VMZs, but until now no protein tested, either with or without mesoderm-inducing activity, has been able to do this.

In two recent papers, Smith and Harland^{15,16} published the results of an expression cloning operation in which a library prepared from lithium-treated, and therefore hyperdorsal, embryos was used to isolate single complementary DNAs with the capacity to induce secondary axes when injected into the ventral side of early cleavage-stage embryos. This is not, in fact, an assay for organizer function, but rather an assay for the dorsal mesoderm-inducing activity that normally results in the formation of the organizer. They succeeded in isolating two cDNAs with high levels of axis-inducing activity. The first was *Wnt-8*, which is normally expressed during gastrulation in the ventral marginal zone and thus is not a good candidate for any inducing signal known so far. The second encodes a novel type of secreted cytokine which they called *noggin*. This was expressed in the organizer during gastrulation, and later in the notochord and prechordal plate, which are the tissues normally formed by the organizer. Although *noggin* is also expressed uniformly at a low level in the maternal messenger RNA pool, the zygotic expression seemed more interesting as it is exactly where we should expect to find the dorsalizing signal.

In their new paper², the same group goes on to show that *noggin* does indeed have dorsalizing activity. The protein can induce muscle from isolated VMZs at a stage when activin and other mesoderm-inducing factors are ineffec-

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tive. Also, an expression plasmid bearing the *noggin* gene under the control of a β -actin promoter can partially rescue a dorsal axis in embryos made uniformly ventral by early irradiation with ultraviolet light. The promoter is not active until the mid-blastula transition, and so active levels of protein are unlikely to have accumulated until gastrulation. This also suggests that the activity is exerted during and not before gastrulation. This story should warn us that the biological activity displayed in the original isolation of a molecule may not be its only or most important activity.

It is now generally recognized that three types of condition have to be met by any substance claimed to be an inducing factor. These can be summarized as: expression, activity and inhibition. Expression means that the substance must be secreted by the signalling tissue at the right embryological stage and in a quantity adequate to do its job. Activity means that the substance should display an appropriate biological activity, preferably in an *in vitro* system so that the 'adequate quantity' can be established. Inhibition means that if the substance is taken away *in vivo*, either by mutation or by some biochemical inhibition, then the induction should fail.

Smith *et al.*² enable us to say that *noggin* has passed the activity test. The previous expression study shows appropriate expression at the RNA level. The protein pattern now needs to be studied and it will be particularly interesting to find whether, as one might predict from the embryology, *noggin* protein is distributed in a dorsoventral gradient. Most important, a convincing inhibition experiment must be performed to show that *noggin* is actually needed for dorsalization *in vivo*. This may prove difficult as targeted mutagenesis is not possible in *Xenopus*, the antisense method has so far been successful only for maternal genes¹⁷, and the dominant negative method, so successful for inhibition of fibroblast growth factor and activin function, requires knowledge of the structure of the receptor.

Assuming that *noggin* does hold up as the dorsalizing signal, what are the implications for the future? First, it opens the way for a detailed study of dorsoventral patterning. We should find out whether *noggin* is itself a morphogen, or whether it lifts an inhibition normally repressing muscle and kidney formation in the ventral region. Doubtless there is also a whole battery of *noggin*-inducible immediate-early mesoderm patterning genes which will now be fished out by differential screening. Second, it will be interesting to find the receptor for *noggin* and to look at the signal transduction pathway. In 1958, Ogi reported¹⁸ that various salt solutions could dorsalize

THIS month (13 February) is the 250th anniversary of the birth of Joseph Banks, a man born to a fortune who used it to good effect. Banks is best known as a botanist and as an indefatigable enthusiast for voyages of scientific exploration, both as participant and sponsor. He journeyed, for instance, to Newfoundland and Iceland. But these were side shows compared with the epic voyage of the Endeavour under James Cook (1768–71), which put New Zealand and east-coast Australia on the map and resulted in a vast haul of botanical, zoological and ethnographical specimens. In this portrait, by Benjamin West, he is seen on his return, self-confidently displaying some of the artefacts gathered in the South Seas. Throughout his long life (he died in 1820), Banks had

fingers in a multitude of pies. His influence was enormous — principally as president of the Royal Society, a job which he held for 42 years, and as a friend of George III, and generally as a fixer and mover in scientific, trading and agricultural ventures. The scale of his activities is reflected in his correspondence, which was dispersed after his death but copies are now being reassembled by the Banks Archive at the Natural History Museum in London. The Royal Society will host a birthday party, in the scholarly form of a conference entitled *Sir Joseph Banks: A Global Perspective*, on 22–23 April. Details of the meeting are to be had from Rex Banks at The Natural History Museum, Cromwell Road, London SW7 5BD, UK.

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Triturus VMZs. The only one of these to work on *Xenopus* is zinc chloride. Although not very specific, Zn is now known to be an inhibitor of tyrosine phosphatases, so we might tentatively predict that the *noggin* receptor will be a tyrosine kinase. Third, we can be sure that *noggin* will be isolated and its function investigated in other vertebrates. People are often rude about *Xenopus* because of its lack of usable genetics. But *Xenopus* goes on producing break-

throughs in developmental biology and, doubtless even as you read this, there are scientists busily cloning *noggin* out of the mouse and the zebrafish, those organisms so eminently favourable to the discovery of novel molecules by genetic means. □

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Long shadow

VOLCANIC eruptions may cast a shadow over marine plankton, says A. E. Strong (*J. geophys. Res.* **98**, 985–987; 1993). In 1982, after the explosive eruption of El Chichón in Mexico, satellite monitoring of the colour of the Arabian Sea showed that the regular summer phytoplankton bloom was much smaller than in previous years. The southwest monsoon that year was also unusually weak. Strong suggests that the dense pall of volcanic aerosol, cutting out sunlight, could have had a twofold effect on plankton: cooler land temperatures could have caused the weaker monsoon, with knock-on effects on ocean currents and nutrient supply; and the reduction in sunlight could have directly affected photosynthesis. In July 1991, Mount Pinatubo hurled still larger quantities of aerosol into the stratosphere. The consequences of that eruption on plankton are not yet clear.

Monster mash

STARBURST or monster — this was the choice offered two years ago B. J. Jarvis and J. Melnick, discussing the violent nucleus of the galaxy M87. Where those astronomers favoured a compromise — a bit of both — J. Braine and T. Wikland plump for just the latter (*Astr. Astrophys.* **267**, L47–L50; 1993). The earlier authors had found spectroscopic evidence of a dense cluster of hot young stars in the galactic core. But Braine and Wikland looked for the molecular gas from which the stars would have formed, and found none. This rather undermines the idea that molecular gas both provides the unseen mass known to be pulling the galaxy's stellar population into the core, and fuels its brilliant radiation. Instead, the case for a massive black hole, the monster, at the galactic centre seems stronger than ever.

Boring defeat

A TEAM at CIBA–GEIGY report successful field trials on maize expressing a synthetic gene for part of an insecticidal δ -endotoxin from *Bacillus thuringiensis* (M. G. Koziel *et al.* *Bio/Technology* **11**, 194–200; 1993). Transgenic plants were produced by microrprojectile bombardment. Hybrids with commercial lines were then created, and were tested in Illinois for resistance to the European corn borer, a severe pest in the mid-west of the United States. Each of the transgenic hybrids was subjected to the attentions of 2,400 larvae over a period of eight weeks, but shrugged off the challenge of both the first- and the second-generation insect infestations. In passing, the authors remark that pest resistance to δ -endotoxins in plants may evolve in time — this is a topic which R.M. May discusses in News and Views next week.

Theories on the brain

Charles F. Stevens

WE still do not have what could be called a theory of the brain. But the upsurge of interactions between theorists and experimentalists, as manifested at a meeting* late last year, offers the optimistic hope that one is on the way.

The interests of the 18 participants — about half of them theorists and half experimentalists — reflected the current state of brain theory, although not necessarily what it should be: cortical function took centre stage, with hardly any mention of theories about structure of neuronal circuits, and the emphasis was on the visual system. The theoretical approaches (as represented at the meeting) fall into four distinct but somewhat overlapping groups. The first is artificial neural networks. Here there was presentation of an analysis of motion detection using a standard network approach, with the additional feature of an executive network that decides which elements of the motion detector deserve attention (T. Sejnowski, Salk Institute), and description of a network that constructs a three-dimensional representation of an image from a few particular views with an interpolation technique that is related to the morphing used to create dramatic special effects in motion pictures such as *Terminator II* (T. Poggio, MIT).

The second category involves relating neural architectures to particular jobs the brain must accomplish, as exemplified by analyses of pattern recognition in terms of requirements of neural architecture (D. Van Essen and C. Anderson, Washington University; S. Ullman, Weizmann Institute; D. Mumford, Harvard); Van Essen and Anderson also use a neural network as an existence proof for the feasibility of their proposed architecture. Here the stress is on the importance of architectures that use feedback from higher to lower levels, as opposed to the strict hierarchical architectures favoured by some earlier workers, with relation of theory to the forward and backward neural pathways that interconnect various cortical areas involved in vision.

The third type of theories are those centred on some unifying notion. Hence the proposal (W. Singer, Max Planck Institute for Brain Science, Frankfurt) that the existence of synchronized firing across populations of neurons is a neglected but important organizing principle of neural information processing, and the argument (H. Barlow, University of Cambridge) that the cortex compares its

input with stored models of previous inputs and separately processes the consequences of the match with the 'new' (that is, the aspects of the input that do not match the model). There is too the attraction of focusing on attention and temporary memory as possible key features necessary for our awareness of sensory events (C. Koch, Caltech; F. Crick, Salk Institute).

The final theoretical approach (my own, 1/18 of all theories) makes use of standard continuous mathematics to describe the computations carried out by cortex: truth to tell, most participants were not enthusiastic about the argument that the properties of synapses and the structure of neuronal circuits lead naturally to a mathematical formalism much like that used by the field theorists in physics.

Clearly, though, there are some distinct challenges for experimentalists in all this, not least in looking at selective attention and the necessity of routing information selectively from one place to another. As Van Essen put it, "How can we write our name with our hand, our foot or our nose?". Some central motor program can be routed to various parts of the motor system, but the experimentalists have failed to provide a mechanism to accomplish this. Most of the proposals for how attention and information routing might be achieved — other computations the brain must carry out require this as well — involve multiplication of one signal by another. But, again, the experimentalists have yet to demonstrate how this is achieved in any specific cases.

Then there is the question of how many neurons are necessary to carry out a particular task, an issue which arises in several contexts. How widely distributed are our representations, and how sharply tuned are the receptive fields of cortical neurons? If only a few neurons at higher levels of the visual system are involved in representing the image of grandmother, say, then neuronal noise becomes a serious problem. But making the connections needed to recognize a complex image (grandmother in a red hat) that is represented by large and only partially overlapping neuronal populations (grandmother versus red versus hat) presents, as Barlow stressed, an even more serious problem. The experimentalists have the tools to find out how many neurons are actually involved in simple representations — equivalently, how broadly tuned the receptive fields are at higher levels of the visual system, for example. The answer will be

*Large Scale Neuronal Theories of the Brain, Idyllwild, California, 9–13 December 1992. Proceedings to be published later this year by MIT Press.

crucial for future thinking about what mode of operation the cortex employs.

Experimentalists also offer a variety of approaches to define the constraints on any theory of the brain. Thus a survey of anatomical information to count the number of brain areas, more than 30 of them, devoted to vision (Van Essen). Multiple simultaneous single-unit recording and computer methods (cross correlation and spectral analysis) have been used to identify synchronous activity at widely separated areas of the visual cortex when the neurons involved are stimulated by a coherent stimulus; these data form the basis for a proposal that synchronized activity is a critical signal (Singer). Also intriguing are records from inferotemporal cortex in waking monkeys presented with complex visual stimuli, which show that the firing of neurons in response to a stimulus is markedly diminished for each stimulus presented after the first, but not for interspersed novel stimuli (R. Desimone, NIH); and, more generally, the application of PET (positron emission tomography) and scalp electrical recording from several sites during complex

tasks performed by humans to identify the brain areas involved in attention (M. Posner, University of Oregon).

So, in turn, experimentalists are providing challenges for the theorists. How does one construct a reliable nonlinear network from populations of very unreliable elements? Why do we need the number of distinct but mutually interacting cortical areas we actually have for visual image processing? How is synchronous firing achieved across a neuronal population and what is its significance? How do we construct the complex receptive fields found in inferotemporal cortex and how can the circuits remember a stimulus so well after just one presentation? How do we shift attention? These are daunting questions, but not cause for despair. To my mind we are going to have a good theory of how the brain works — if theorists and experimentalists can sustain and expand collaborative work. □

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NUCLEAR PHYSICS

A shattered halo

P. G. Hansen

THE discovery five years ago of a lithium isotope with a tenuous 'halo' of neutrons that extends far beyond the core of the nucleus opened up a rich new vein of nuclear physics with intriguing links to the physics of loosely bound quantum systems. New experiments^{1,2} on the momentum of the debris from break-up of the halo show that it has an elegant simplicity, without denying the prospect of further subtle effects showing up under more detailed examination.

The isotope in question is ^{11}Li , consisting of just three protons but eight neutrons, the last two of which form the halo. As the lighter isotope ^{10}Li , with one less neutron, decays instantly into ^9Li and a neutron, it is clear that the attractive neutron-neutron force is essential to the stability of ^{11}Li , which can be viewed as a ^9Li core coupled to a pair of planetary neutrons.

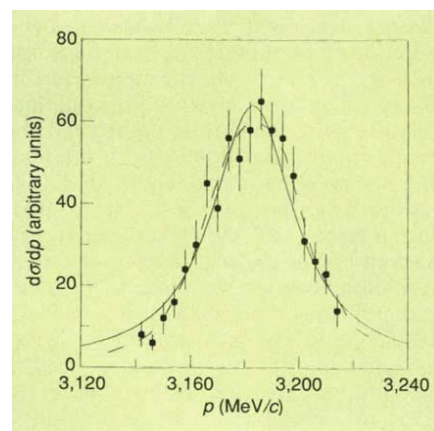
As I mentioned in a News and Views article³ when the species was first discovered, the halo owes its existence to a basic quantum-mechanical phenomenon — the tunnel effect, which allows the weakly bound halo neutrons to get far from the core and far from one another. Most tunnels lead from one place to another (the tunnelling associated with some types of radioactivity is like this), but in this case the analogy is closer to

the tunnel in a mine, which leads to a dead end. Speaking more formally, quantum mechanics allows the neutrons to have negative kinetic energy in parts of space — the halo.

Quantum mechanics also links the size of the halo, through Heisenberg's uncertainty principle, to the momenta of the constituents, which is the easier quantity to measure. This is what has been done in the pair of new experiments at Michigan State University^{1,2}, looking at the ^9Li fragments and neutrons produced by the dissociation of ^{11}Li .

Not that the experiments are straightforward. First, the radioactive ^{11}Li isotopes have to be made by slamming ^{18}O ions into a beryllium foil. They then have to be selected from the debris of the collision by magnetic analysis and directed at a second metal target. The intensity of the secondary beam is a mere 300–1,500 ^{11}Li atoms per second, depending on the requirements.

Both experiments observed the debris from reactions of the secondary beam with the second target. If this is chosen to be a heavy metal (tantalum or lead), then the dissociation of ^{11}Li is caused essentially by the electric field which pushes the ^9Li core away from the neutrons. On the other hand, a light target such as beryllium interacts through the



The parallel momentum distribution of the ^9Li recoils measured¹ at 0° and at a beam energy of 66 MeV per nucleon. The full drawn line is a one-dimensional Lorentzian with $\Gamma=37$ MeV/c and the dashed line a Coulomb-excitation calculation due to Esbensen and Bertsch.

nuclear force with the two halo neutrons in such a way that they are deflected away from the core. This process resembles the bending of light around a small obstacle and the mechanism is referred to as diffraction dissociation.

The experiment of Orr *et al.*¹ measured the change in momentum of the outgoing ^9Li fragments parallel to the beam direction. As this quantity is much smaller than the actual momentum spread in the beam it was necessary to apply a trick that makes the spectrometer, composed of two analysing magnets, insensitive to the absolute velocity of the in- and outgoing lithium ions but sensitive to changes in this quantity caused by the reaction in the second target. The figure shows the parallel momentum distribution deduced from such data. Note that nuclear physicists prefer to measure energies in megaelectronvolts (MeV) and velocities in units of the speed of light, so that the appropriate unit for momentum is MeV/c.

It is convenient in the first instance to assume that the central part of the momentum distributions can be approximated as a bell-shaped Lorentzian curve, characterized by a single parameter Γ . This is simply the measured full width at half maximum, only 37 MeV/c. The very small value is evidence for a large spatial object, the halo, which must have a radius around five times larger than that of ^9Li . A second striking feature is that the beryllium target gave almost the same measured width although the reaction mechanisms are different in the two cases. Together with other evidence, this suggests that the momentum distribution is at least approximately representative of that existing in the radioactive projectile before the dissociation took place.

For the electrical break-up of ^{11}Li on a lead target, Ieki *et al.* (in the latest

*Physical Review Letters*²) measured the directions and energies of both neutrons and the energy of the core recoil, a so-called kinematically complete measurement. They found that very low excitation energies dominate in the final three-body system and argue that it is neither necessary nor desirable to attribute a strong collective character to this transition. With normal nuclei, electrical excitations connect by collective (multi-particle) transitions of the whole nucleus to excitation energies around 25 MeV, so-called giant dipole resonances. But in the lithium case, the authors prefer to view the process as predominantly a direct break-up leading to free single-particle states with low kinetic energy, of the order of 1 MeV or less.

The two experiments also contribute towards resolving the question of correlations, whether the neutrons move independently of one another or whether they are coupled somehow on opposite sides of the core, say. Already the first measurements⁴ of neutron-neutron coincidences were consistent with the assumption of no correlations between the momenta of the neutrons, and the new experiments confirm this with much higher precision. The authors find that their reconstructed decay-energy spectrum can explain their data as well as the previous transverse-neutron⁴ and parallel-core momentum¹ distributions, all with the assumption "that the decay energy is shared by ${}^9\text{Li}$ and the neutrons according to 3-body phase space"; in other words, neutron-neutron correlations are absent.

Comparisons

It is interesting to see how these conclusions fit with measurements of transverse momentum distributions of neutrons and core recoils. The ${}^9\text{Li}$ recoil momentum distribution in the break-up on light targets was found at the Berkeley Bevalac to be characterized by $\Gamma_R=90$ MeV/c. The angular distribution of the neutrons measured at GANIL in Caen gave $\Gamma_n=26$ MeV/c, both for heavy and for light targets. Comparison of the two values shows that they cannot represent the same intrinsic momentum, as the total momentum would not then be conserved, even if the two neutrons were moving in parallel. The momentum balance can be saved only if rather arbitrary additional assumptions (two-component momentum distributions) are made, as has been done in a recent paper by Tanihata *et al.*⁵, who suggest that the two neutrons move essentially in parallel. If on the other hand the neutrons are assumed to be uncorrelated, then the recoil momentum width for the pair suggested by the GANIL experiment is $\Gamma_n\sqrt{2} = 37$ MeV/c, in exact agreement with the new result.

It is not completely surprising if the momentum component of the ${}^9\text{Li}$ recoil parallel to the beam axis is more representative of the situation before the collision than is the transverse component. This is known empirically for certain nuclear break-up process at high energy. And it is also easy to see that for electrical break-up the parallel momentum component to a first approximation is unchanged because the retardation caused by the Coulomb repulsion on the incoming leg of the trajectory is compensated on the outgoing leg. It is tempting to assume that the transverse core-recoil distribution is broadened by some mechanism that we do not yet fully understand.

Reactions

Finally, the two new papers both take up a subject that could be of great diagnostic value for understanding in more detail the reactions of halo nuclei, namely that of possible shifts in the average fragment velocities relative to the beam velocity. The following simple model illustrates one mechanism that could cause such shifts. Consider ${}^{11}\text{Li}$ passing a heavy target in a straight-line trajectory. At the minimum distance the system has increased potential energy due to the electrostatic repulsion of the two nuclei and the kinetic energy is reduced by the same amount. If break-up takes place at this point, then the neutrons will emerge from the reaction with a proportionately reduced energy. The charged fragment, on the other hand, regains the missing energy on the outgoing leg of the trajectory, but as it is lighter than the projectile this will lead to a velocity slightly higher than that of the beam. Qualitatively, at least, effects of the expected order of magnitude have been observed for the neutrons⁴, the charged fragments¹, and for the neutron-core velocity difference².

The two Michigan papers represent an important milestone towards a qualitative understanding of the two-neutron halo. The operative word here is clearly qualitative. We definitely expect effects such as neutron-neutron correlations, collective contributions and resonances to make their appearance at some improved level of experimental precision, but for the moment we are not in great need of them, which should please those who believe that a simple picture is preferable to a complicated one. □

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DAEDALUS

Kiss and tell

THE human skin is home to a vast variety of microorganisms. These harmless yeasts and bacteria feed on the many nutrients that it secretes, and shelter in its many niches, from hair follicles to sweat glands.

Each region of your skin (your hands, say, or your lips) has its own mixture of species and strains, continuously selected and maintained by the local conditions, and each as individual as a fingerprint. Somebody else's hands or lips, with different physical and chemical conditions, will support quite a different ecology. So a handshake or a kiss brings two different microscopic worlds together. Migrants pass in both directions; the ecology of each skin surface is challenged by an influx of competing newcomers. The natives are better adapted to the local conditions, but they may take many days gradually to oust the colonists and restore the previous ecology. So, says Daedalus, a good bacteriologist could tell who has kissed whom where, and when.

A DREADCO team is now applying this awesome ability, not merely to kisses and handshakes, but to hugs, punches, assaults and to various forms and degrees of sexual intimacy. The technique should be very sensitive. The skin can carry dozens of organisms, each in dozens of strains. Each could be positively identified by DNA tests; but selective culturing and testing with depleted media should be just as good and far easier. Furthermore, skin bacteria reproduce and maintain themselves. Even washing doesn't dislodge them; the survivors rapidly multiply back to their original density.

A husband and wife in continual contact, for example, might come to hold many organisms in common. A brief outside dalliance by one of them would introduce new organisms into the menage; these would spread slightly to the innocent spouse before being gradually eliminated by the indigenous ecology. By identifying the organisms and their changing counts on the various parties, DREADCO could establish when the dalliance occurred and with whom. Washing or bathing could not destroy the evidence.

A great deal of personal conflict and civil and criminal legal wrangling arises from allegations of various sorts of bodily contact. Daedalus will soon be in a position to prove or disprove them. Already DREADCO is being besieged by desperate detectives, outraged wives, victims of violence or molestation, maligned politicians and indignant good friends, all clamouring for proof that somebody is guilty, or that they themselves are innocent, of something. Daedalus seems sure of a profit on his research investment.

David Jones

Which gp160 vaccine?

SIR — The lobbying efforts by MicroGeneSys Inc. (MGS) to win 'pork-barrel' funding from the US Congress for its HIV-1 gp160 vaccine candidate have attracted much criticism^{1,2}. Little attention has, however, been paid to the actual product, which is a baculovirus-expressed, recombinant version of the outer envelope glycoprotein precursor gp160 from the HIV-1 LAI isolate.

Many companies and research laboratories have produced gp160 or its de-

Because of the predominantly denatured state of MGS gp160, it does not detectably bind neutralizing, human monoclonal antibodies to discontinuous or conformationally sensitive gp120 epitopes (G. K. L. *et al.*, manuscript in preparation). Neither have we been able to isolate murine hybridomas specific for such epitopes after immunization with MGS gp160; all the antibodies recognize linear epitopes and most bind poorly, if at all, to native, recombinant gp120.

Antibodies against conformational or discontinuous epitopes of gp120 are far more prevalent than those to linear epitopes in HIV-1-infected people and are responsible for much of the neutralizing activity in serum⁴⁻⁶. Thus MGS gp160 is unable to mimic the native glycoproteins normally seen by the human immune system. Furthermore, because the MGS protein is derived from the LAI sequence, its principal neutralizing V3 domain is not representative of the types of virus circulating in the population⁷. Consequently, MGS gp160 induces V3 loop antibodies capable of cross-reactivity with more representative V3 loops only very inefficiently⁸. Moreover, inbred strains of mice immunized up to six times with MGS gp160 lacked any serum-neutralizing antibodies, in marked contrast to their responses

to native gp120s from other manufacturers⁸. Other studies with denatured envelope glycoproteins have confirmed their inferior immunogenicity compared with native forms^{9,10}.

It is by no means certain that immunotherapy with envelope glycoproteins will be beneficial to its recipients; available results from pilot studies with MGS gp160 appear to be disappointing^{11,12}. But if social and political pressures force the undertaking of large-scale clinical trials, it is crucial that they do not focus only on a single product. Better quality native gp120 or gp160 proteins are available from several other sources, in some cases with a V3 loop sequence more relevant than that of HIV-1 LAI (refs 10,13).

We do not know what form of envelope glycoprotein is best for use *in vivo*. The optimal properties of a recall antigen or an inducer of cellular immun-

ity may differ from those of a stimulator of a *de novo* antibody response. However, uptake of gp120 into monocytes for processing and antigen presentation can be a CD4-dependent process¹⁴ which presumably is most efficient with a native form of gp120. To determine these factors, different immunogens would need to be evaluated in small-scale comparative studies. However, *in vitro* analyses and immunogenicity studies in small animals do have a bearing on the ability of MGS gp160 to stimulate humoral immunity. The information described here strongly suggests that the ability of MGS gp160 to induce the production of relevant antibodies is severely limited by its properties. Its failure to act as an efficient inducer of neutralizing antibodies in mice bodes ill for its likely activity in humans.

Although broadening the immune response to HIV proteins may be useful¹⁵, it is not clear to us what is the importance for humoral immunity of antibodies raised to a denatured protein that are unable to recognize native viral glycoproteins. In short, based on existing *in vitro* data, our opinion is that there could not be a worse choice from the current envelope glycoprotein vaccine candidates than MGS gp160 to stimulate at least one important arm of the human immune system, the production *de novo* of cross-neutralizing antibodies to the V3 loop and discontinuous epitopes around the CD4-binding site.

John Moore

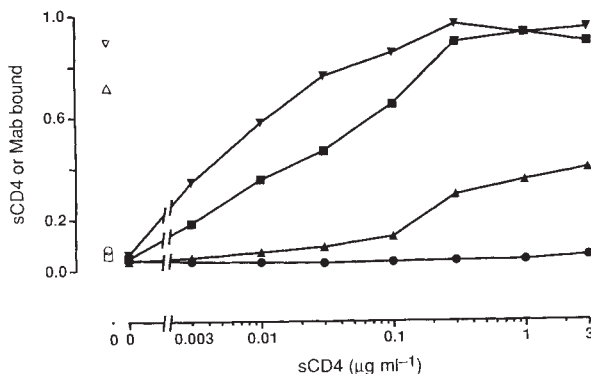
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Binding of sCD4 to different recombinant gp120/gp160 preparations. gp120 (0.1 $\mu\text{g ml}^{-1}$) or gp160 (0.5 $\mu\text{g ml}^{-1}$) in TBS containing 10% FCS and 1% NP-40 nonionic detergent was captured onto a solid phase via sheep antibodies to the last 15 amino acids of gp120 (ref. 3). sCD4 was titrated against each gp120 and bound sCD4 (closed symbols) was detected with anti-CD4 antibodies³. The presence of gp160 on the solid phase was confirmed using human monoclonal antibody 50-69 to the immunodominant region of gp41 (open symbols), a gift from S. Zolla-Pazner. No gp120/gp160 ($\square$, $\blacksquare$); Celltech CHO-expressed LAI gp120 ($\square$, $\blacksquare$); MGS baculovirus-expressed LAI gp160 ($\triangle$, $\blacktriangle$). Smith-Kline Beecham Biologicals vaccinia-expressed LAI gp160 (∇ , $\blacktriangledown$). The last two proteins were obtained from the UK MRC AIDS-Directed Programme. We tested two batches of MGS gp160; data from that giving the stronger sCD4 binding are shown.

native gp120 for research or clinical purposes, including American Bio-Technologies Inc., Celltech Ltd, Chiron Inc., Genentech Inc., Immuno AG, Repligen Inc., Smith-Kline Beecham Biologicals SA and Transgene SA. Without exception, their products are predominantly native glycoproteins which bind CD4 with high affinity. Uniquely, MGS has made a gp160 molecule which is misfolded and substantially denatured and which binds CD4 very poorly compared with others (see figure). The residual CD4 binding to MGS gp160 probably reflects the presence of a small percentage of native molecules in the product, rather than a homogeneous product of low affinity for CD4, although we cannot be certain of this. If so, we estimate that the active fraction is considerably less than 1% of the total. Similarly, the gp120 sold by MGS binds CD4 extremely poorly³.

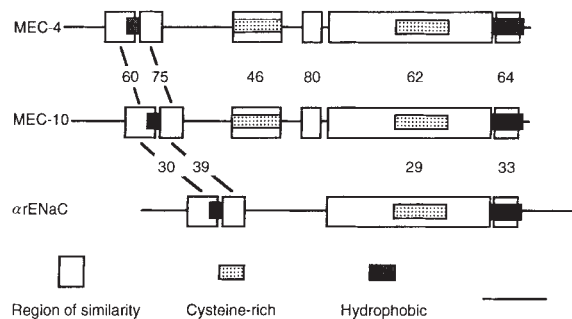
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Degenerin similarities

SIR — We were delighted to hear, before publication on 4 February, that Canessa *et al.*¹ had identified a gene whose product, α ENaC, conveys amiloride sensitivity on *Xenopus* oocytes and exhibits extensive similarity to two *Caenorhabditis elegans* genes, *deg-1* (ref. 2) and *mec-4* (ref. 3), that can mutate to produce neuronal degeneration. We have called the products of these *C. elegans* genes degenerin³. Here we would like to point out that the similarity extends further than what is evident from our previously published sequences, demonstrating that the mammalian and *C. elegans* genes are members of the same gene family.

We have been able to obtain full-length coding sequences of the *mec-4* gene and of a new member of the degenerin family, *mec-10* (see figure). The sequences predict proteins of 768 and 724 amino acids, respectively. The *mec-10* protein has the two cysteine-rich domains and carboxy-terminal hydrophobic domain previously noted in the *mec-4* protein sequence³. In addition, the *mec-4* and *mec-10* proteins have a second hydrophobic domain approximately 100 amino acids from the amino-terminus and regions of similarity both before and after this putative membrane-spanning region. This more amino-terminal hydrophobic region and the associated conserved sequences are also found in the rat α ENaC protein¹. Thus, all three proteins have a similar structure and exhibit regions of similarity over their entire lengths, indicating that they are members of the same gene family (although the rat protein does not have the more amino-terminal cysteine-rich region).

The finding that α ENaC can convey amiloride-sensitivity to *Xenopus* oocytes raises the intriguing possibility that *mec-4*, *mec-10* and, presumably, *deg-1* also encode subunits of a similar sodium-channel complex. This is particularly exciting because *mec-4* and *mec-10* were first identified by virtue of mutations that caused *C. elegans* to be touch-insensitive^{4,5} and because these genes



Comparison of the predicted protein sequences of MEC-4, MEC-10, and α ENaC. Regions of similarity have at least 25% sequence identity compared to MEC-10; exact values are indicated in the figure. MEC-4 and α ENaC share the same four regions of similarity that MEC-10 and α ENaC do (the corresponding percentages of identity, starting at the amino termini, are 30, 36, 23 and 31). The α ENaC sequence was generously provided by B. Rossier before publication¹. Scale bar, 100 amino acids.

have been hypothesized to be part of the mechanosensory transduction machinery^{3,5}. These genes (and the *mec-6* gene, which is needed for touch sensitivity^{4,5} and for the *mec-4* and *deg-1*-dependent degenerations²) may be part of a mechanotransducing channel.

It is likely, given the fact that several members of this family are found in *C.*

elegans, that families of these genes will be found in other organisms. Since several mechanosensory hair cells (for example, in the lateral line organ of *Necturus*⁶ and in the vestibular organ of chick⁷) as well as a mechanosensitive channel in *Xenopus* oocytes⁸ are sensitive to amiloride, mechanosensory channels in other organisms may use degenerin-like genes. It will be interesting to see whether these new proteins are channel components, are involved in mechanosensory transduction, or cause cell degeneration, either naturally or through mutation.

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Sex surveys and drug users

SIR — The British national survey of sexual attitudes¹ and the French national telephone survey² provide general population norms for sexual behaviour, giving a control group “against which research in more focused populations at high risk (of HIV) . . . can be assessed”¹. One such population is injecting drug users, who are recognized as a source of heterosexual spread of HIV infection. While it is widely believed that drug users who take depressants (for example, opiates and tranquilizers) practise less sexual intercourse than the

norm³, such assertions have been at best speculative because it has been difficult to relate such behaviour to general population norms.

Between May 1990 and December 1991 (fortuitously identical to the time period of the British survey), 919 of Glasgow’s estimated 9,400 injecting drug users were investigated⁴. A multicentre and community-wide sampling strategy was implemented to provide as representative a sample as possible of injecting drug users in Glasgow. Respondents, of whom 88% injected daily and 99%

NUMBER OF HETEROSEXUAL PARTNERS BY AGE AND GENDER

	Men				Women			
	16–24		25–34		16–24		25–34	
	Glasgow	Ref. 1	Glasgow	Ref. 1	Glasgow	Ref. 1	Glasgow	Ref. 1
None	16.1%*	26.9%	17.6%*	8.6%	19.6%	23.9%	27.6%*	6.7%
One	46.1%	46.2%	52.7%*	76.9%	62.7%	60.5%	58.6%*	86.8%
Two	12.7%	14.3%	10.1%	8.6%	8.9%	10.0%	8.0%	4.9%
3–4	16.4%*	9.1%	10.5%*	4.1%	7.6%	4.5%	3.4%	1.3%
5+	8.6%*	3.5%	9.1%*	1.9%	1.3%	1.0%	2.3%	0.3%
99th centile	12	10	20	5	5	7	5	3
Mean	1.9*	1.4	2.0*	1.2	1.1	1.0	0.9	1.0
Variance	4.7	5.2	13.3	8.9	0.9	1.8	0.7	0.3
N	347	1,984	296	2,167	158	2,246	87	2,899

The table is a comparison between the study reported here (Glasgow) and the British survey (ref. 1). Number of partners are in the past 6 months for the Glasgow study and the past year for ref. 1. Sexual activity resulting from prostitution was excluded from the Glasgow study.
* Significant difference between the Glasgow study and ref. 1 ($P < 0.05$)

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were currently injecting either opiates or tranquillizers, were asked about their sexual behaviour (see Table).

Despite the shorter time period of analysis, the mean number of partners of male drug users in both age bands was significantly greater than that of the general population. Male drug users were also more likely to have had three or more heterosexual partners. In contrast, female drug users reported a similar mean number of partners in each age band to the British survey¹. The crucial message is that, for both sexes, drug users have no fewer sexual partners than the corresponding general population controls.

Of course, a similar or greater number of sexual partners does not necessarily mean increased sexual activity but, with respect to HIV transmission, it does represent increased sexual mixing and therefore greater potential for dissemination of infection. The frequency of sexual intercourse among injecting drug users cannot be compared with the Brit-

ish survey data¹ since this has yet to be published. Nevertheless, during a 4-week period, male and female respondents (age 18–34) in the French survey² reported 8.7 and 8.6 episodes of sexual intercourse, respectively, while male and female drug users in Glasgow (matched for age) reported 10.2 and 9.6 events.

These findings demonstrate that drug injectors with serious opiate and tranquillizer dependency are just as sexually active, if not more so, than the general population.

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Satellite ozone monitoring error

SIR — The total ozone mapping spectrometer (TOMS) aboard the Nimbus-7 satellite gave total ozone amounts a few per cent too high during its annual calibration comparison with the world standard ozone spectrophotometer (Dobson 83) at Mauna Loa Observatory, Hawaii, during the summer of 1992. The immediate significance of this error is that the geographical coverage of the 1992 spring ozone minimum over Antarctica, which was of record size,

may have been slightly larger than previously announced¹, and that TOMS measurements of the ozone column within the minimum were somewhat too high.

The new problem was first suspected last spring when it was discovered that TOMS ozone observations were consistently higher than those made by two TOPS (total ozone portable spectrometer) filter ozonometers² at Seguin, Texas (29° 35' N 97° 58' W) (see figure).

Although the mean of TOPS observations during all of 1991 was only 0.02% higher than the mean of TOMS observations, from 1 February to 30 September 1992, TOMS gave ozone amounts 3.7% higher than the mean of TOPS observations.

It was first thought that the inconsistency was caused by volcanic haze, but when the TOMS ozone observations for Mauna Loa Observatory, Hawaii, for 3–11 August 1992 were received in early November, the mean ozone amount given by TOMS was 1.7% higher than the mean of the corrected Dobson 83 measurements for the same period. This is twice the previous greatest difference (1988) (ref. 3) and nearly three times the standard deviation of the

1979–91 series of comparisons (0.6%). It is also nearly twice the annual calibration drift of the instrument (0.9%)⁴ and is in close agreement with an approximately 2% difference between Nimbus-7 and Meteor-3 TOMS instruments (R. McPeters, personal communication). Similar differences between ozone observations by the Nimbus-7/TOMS and both Dobson and balloon ozonesonde measurements from Antarctica have recently been informally reported.

The Nimbus-7, which has completed more than 70,000 orbits since its launch in 1978, has greatly exceeded its predicted lifetime. The only other TOMS instrument now in orbit, the Meteor-3/TOMS, is in a non-Sun-synchronous orbit, and its data are not yet considered validated. Several years ago there were suggestions that the Dobson ozone network was no longer necessary in view of the global coverage provided by the Nimbus-7/TOMS. The new calibration problem demonstrates both the wisdom of NASA's continuing comparison of satellite ozone observations with Dobson 83 and the importance of maintaining a carefully calibrated, ground-based ozone monitoring network³.

Forrest M. Mims III

Science Probe, Inc.,

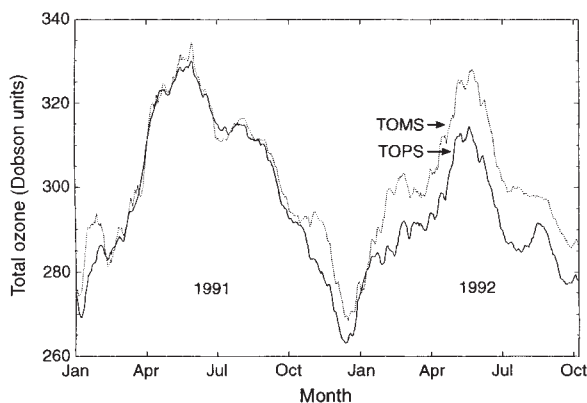
433 Twin Oak Road,
Seguin, Texas 78155, USA

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Thirty-day running mean of nearly simultaneous total ozone observations by TOMS and TOPS-1, a two-wavelength (300 and 306 nm) filter ozonometer. The TOPS-1 ozone reduction algorithm is derived from a comparison with TOMS from September to December 1990. The mean of all TOPS-1 observations during 1991 is 0.02% higher than the mean of all TOMS observations. The mean of all TOPS-1 observations from 1 January to 30 September 1992 is 3.4% lower than the mean of all TOMS observations. Because a comparison of nearly simultaneous ozone observations by two TOPS instruments since July 1990 yields a correlation coefficient (r) of 0.995, it is concluded that the TOPS instruments have not experienced significant drift.

Protein structure prediction

SIR — It has long been recognized that secondary-structure predictions from reliably aligned protein families are more accurate than those from individual amino-acid sequences¹. In their Scientific Correspondence, Benner *et al.*² say that the only true test of predictive power is to apply the method to a protein before its structure determination by experimental methods. This view is shared by others who have predicted the structures of proteins from multiple sequence data and who have had those predictions confirmed subsequently by experimental methods^{3–7}.

Although the details of the prediction methods differ, the unifying theme is the exploitation of the mutation patterns present in multiple sequence data. All such methods require sequences that are sufficiently similar to align accurately, yet show enough divergence to highlight the patterns of conservation. The end result is usually a 'consensus' or 'aver-

age' secondary structure prediction for the family.

The prediction of Benner *et al.* was in the event disappointing in some respects⁸. But other predictions have been better. The recent publication of tertiary structures for SH2 domains from *src* (ref. 9), *abl* (ref. 10) and p85 α (ref. 11), for example, has confirmed our previously published analysis of this family⁷ in which we aligned 67 SH2-domain sequences and used this information to predict the secondary structure and position of buried residues. The prediction was performed by combining two-turn and three secondary-structure prediction algorithms, and was augmented by the identification of residue conservation patterns characteristic of helix, loop and strand. The experimentally determined structures confirm our secondary-structure prediction within the conserved core, although we failed to identify a small surface sheet in a variable part of the domain. The overall accuracy of the prediction on a residue-by-residue basis was 78, 76 and 76% when compared to the secondary structures of *src*, *abl* and p85 α SH2-domains, respectively. Furthermore, analysis of the *src* SH2-domain coordinates⁹ shows that the 22 positions predicted to be buried are inaccessible to solvent.

We, in common with most people developing methods for protein-structure prediction, welcome challenges to predict the structures of other proteins that are soon to be experimentally determined.

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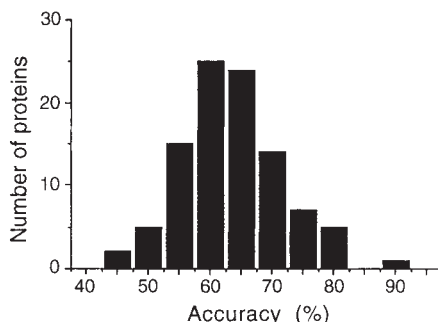
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SIR — Benner *et al.*¹ appear to have fallen foul of the classic mistake of so many architects and analysts of protein secondary-structure prediction. They forget that “one swallow does not make a summer”.

Benner *et al.* consider a single protein

as a test case when comparing protein secondary-structure prediction methods. But all these methods are directly or indirectly statistical in nature, and as such can be assessed only over applications to many proteins. The figure shows the distribution of the quality of results for predictive success using our GOR method², which Thornton *et al.*³ took as the standard comparison when reviewing the method of Benner *et al.* in *News and Views*. The results in the figure are on a three state (α -helix, β -strand and coil) basis for 98 proteins. The fair and proper



Histogram of the accuracy of secondary-structure predictions (α -helix, β -strand and coil) by the GOR method including directional amino-acid pair frequencies². Note that before prediction, each protein is removed from the database for calculating the pair frequencies.

measure of predictive success or accuracy (not always used) is that a residue is either right or wrong in its assignment to one and only one of the three states. Note that there is a broad spread of predictive capability with a standard deviation of the accuracy per protein of 6–8%, depending on the method. Some proteins are easy to predict and some are very difficult, so we emphasize the spread rather than the mean of 65%. Although a level of accuracy of 65% is useful, it could well be that the particular protein of interest is one of those which predict at the 45% level (close to random at 38%), or indeed at 90%!

In attempting to predict the structure of SH3 (a small protein domain homologous to various signal transduction proteins) the dice have fallen badly for Benner *et al.* Our analysis of their prediction shows that they predicted only 46% of residues correctly, and Rost and Sander⁴ found 56% accuracy. Either way, an earlier comparison of the method³ (again on a single protein) seemed to promise a more satisfactory 63%. We have found that most secondary-structure prediction methods (except perhaps those based on homology to other proteins⁴) seem to fare badly with SH3, perhaps a consequence of dominance of three-dimensional ‘tertiary’ effects over local, secondary structure effects.

We agree with Benner *et al.* that if

several homologous proteins are known, secondary-structure prediction based on analysis of the similarities⁵ should bring the average accuracy to much higher values. M. E. Sternberg and others suggested early on that a 4% improvement may be possible, and J. M. Levin (in J. G.’s laboratory) has shown that, providing poorly matching sequences are not included, an average 7% point improvement can be obtained following automatic multiple alignment.

We disagree with Benner *et al.* that other methods are not credible if they are applied after the experimental results are obtained. Statistically, one can and should test performance on the broadest possible number of test cases, providing the method is objective, programmable, fully automatic and well-described in the literature, and providing that the performance in terms of percentage residues correct is based on the (laborious but fair) operation of re-calibrating the parameters or rules with every protein removed from the database when its secondary structure is to be predicted. The GOR methods have survived for more than a decade because of their formal correctness, ease of reproduction and various methods of objective testing. By seeking to incorporate intuition, insight and expertise interactively, Benner *et al.* do not satisfy these criteria, and they admit there is a subjective element⁶. Then, ‘blind’ prediction tests become the only acceptable route, but these then run into the dangers of assessing quality from a very small sample. Reproducibility is the cornerstone of science, and although that may appear hampering to the creative, it does have very considerable benefits.

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■ Another method which may not be widely known to *Nature*’s readers is that of B. V. Shestopalov (*Molec. Biol.*, Moscow **24**, 900–927; 1990; Engl. transl.), which predicts the SH3 domain structure to a similar degree of accuracy as the other methods discussed in this debate. Dr Shestopalov, of the Institute of Cytology, Russian Academy of Sciences, St Petersburg 194064, fax 247 0341, would appreciate receiving protein sequences for secondary structure prediction by his method. □

Nature's semantics

Ian Stewart

Complexity: The Emerging Science at the Edge of Order and Chaos. By M. Mitchell Waldrop. *Simon and Schuster/Viking*: 1992. Pp. 380. \$23 (hbk), £9.99 (pbk).

Complexity: Life at the Edge of Chaos. By Roger Lewin. *Macmillan, New York/Dent*: 1993. Pp. 208. \$22, £15.99.

NEW buzzwords are flying, and both authors manage to get all of them into their title and subtitle: 'complexity', the 'edge of chaos'. Heady stuff. "Vital and controversial", says Stephen Jay Gould in the blurb for Lewin's book; "a deep tale of science in the making", says Douglas Hofstadter on Waldrop's. Is complexity theory really novel and exciting? Does either book capture its idiosyncratic and slightly elusive character?

In brief: yes and yes. The complexity theorists have an important message to convey; and although similar messages have come down to us from previous ages, notably at the urging of some philosophers of science, this particular version of the message locks in more directly with problems of genuine concern for real scientists. An idea mustn't just arrive: everybody has to *know* it's arrived. And both books manage to explain the main concerns and conclusions of the complexity theorists without getting tangled up in fine points. Lewin's book is more journalistic, more of a 'people' book; Waldrop's goes into slightly more depth on the science. Both focus on the Santa Fe Institute in New Mexico, as the first well-known centre specially set up to study complexity (but let us not forget Stephen Wolfram's Centre for Complex Systems). Both leave us wanting to know more, which is the proper function of a popular science book. Both, for journalistic reasons, tell the story from one viewpoint, the complexity theorists', and fail to ask some awkward questions. But what they tell, they tell well; and anyone not brain-dead or hopelessly reactionary should listen.

Complexity, in the sense used here, is not mere complication. It is that curious mix of complication and organization that we find throughout the natural and human worlds: the workings of a cell, the structure of the brain, the behaviour of the stock market, the shifts of political power. The underlying thesis of complexity theory is that traditional reductionism — understanding systems by breaking them down into components and analysing the interactions between them — cannot provide an adequate understanding of such systems. It is the old problem of 'emergent phenomena', of wholes that somehow transcend their parts — indeed of wholes that are to a

great extent *immune* to the detailed structure of their parts. It is about as far as you can get from the 'theory of everything' philosophy espoused by many cosmologists and particle physicists; because — to put it crudely — it holds that stock markets would still crash if the laws of quantum chromodynamics were completely different.

The crux of the argument is that there are three kinds of system: those that don't do anything terribly interesting (steady states, periodic cycles — a lot of classical physics, in short); those that are so complicated that they are just a huge mess, lacking even interesting statistical features (gas molecules, most of the rest of classical physics); and those that hover in between, structured but unpredictable, a flux of almost-patterns (most of biology, economics, politics, sociology, psychology). The three horsemen of the dynamic apocalypse: Death, Chaos and Complexity. Or Death, Chaos and Life, as it is living systems above all that occupy the middle ground, the mysterious 'edge of chaos'.

The common character of complex systems is the interaction of large numbers of reasonably similar components; but the viewpoint is that various large-scale phenomena emerge naturally and predictably within such a context. Many of those phenomena are baffling to any reductionist view: they include self-organization, increasing complication and sophistication, unexpectedly long periods of quiescence, sudden bursts of wild activity. Despite — maybe because of — an immense reductionist attack on the molecular structure and function of DNA, we really know very little indeed about biological development: about form rather than chemistry. As Richard Lewontin recently wrote in *The New York Review of Books* (28 May 1992), the common image of DNA as a self-replicating molecule is about as true as describing a letter as a self-replicating document. The letter needs a photocopier; the DNA needs a cell. Perhaps we should occasionally look at how the photocopier works.

It is not sensible in a short review to try to summarize the arguments for and against this position. The issues are themselves complex: on the one hand, many precursors for such thoughts exist, so the theory can be dismissed as

'nothing new'. On the other hand, if enough scientists went along with it, it would strike at the heart of much orthodoxy. Traditional mathematical economics, for example, with its emphasis on equilibrium and 'rational expectations', would largely be wiped out. This may well be one of the core arguments in favour of complexity theory, because economic events do seem inconsistent with the classical models.

Scientists of a more traditional bent — that is, most of us — would do well to take serious note of the kinds of question that complexity theory hammers home. They should be much less serious about its current answers to those questions. In particular the 'edge of chaos' is at best a partial and metaphorical answer to emergence. I note in passing that our metaphors in this area are getting very confused: 'chaos' used to mean total disorder; then, in the hands of what are now called chaos theorists, it came to mean apparent disorder with a simple, nonrandom cause; but in complexity theory it has once again become total disorder. *Plus ça change*. . .

I also think the mechanism of 'anti-chaos' — that emergence results from the *narrowing* of the range of possible internal states due to adaptation to the environment — is highly questionable. Emergence is surely more a matter of coherent large-scale function riding on and irreducibly driven by an incredibly intricate internal structure, but one that is 'transparent' to the large-scale function. Think of the human visual system. Our brains construct an amazingly effective model of reality, not because their internal structure has become *simplified* as a result of adaptation to the task at hand, but because their 'programming' has become inordinately *complicated*, even though its workings are necessarily hidden from our conscious minds. We can teach computers antichaos, but we can't teach them to see.

The importance of complexity theory, at this early stage in its development, lies in how it shifts the scientific goalposts away from ever-more-detailed analysis of fine internal structure towards a more global explanation of forms, features and functions. Both books convey the message clearly and readably: it is time science relearned how to look outwards as well as inwards, to think about meaning as well as counting information, and to appreciate nature's semantics as well as its syntax. This is the core of the complexity manifesto. Read it, think about it, disagree with it, make television programmes about it, write polemics against it — but don't ignore it. □

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From dock to doc

Len Goodwin

From the Greenwich Hulks to Old St Pancras: A History of Tropical Disease in London. By G. C. Cook. *Athlone*: 1992. Pp. 338. £35.

WHENEVER a nation undergoes a radical change in its technology or its political and military strategy, there are redundancies, often on a massive scale. So it was in Britain at the end of the Napoleonic wars, when the streets of London were crowded with desperate, homeless, workless sailors. Many had

patients had sailed in tropical waters and had diseases such as malaria, cholera, typhoid and dysentery, rarely seen in Britain now that the marshes had been drained and sanitation improved.

The British have been accused of inventing 'tropical medicine' as a speciality — but it was really inevitable in the age of imperialism, when explorers, the military, colonial administrators, industry and finally settlers came face to face with new and unfamiliar diseases in tropical countries. Medical officers in the army and the colonial service (many of them Scots who had difficulty in finding employment at home) occupied their spare time by studying the diseases and the life cycles of the parasites that caused them.

The most famous was Patrick Manson,

based in the London School of Hygiene and Tropical Medicine, which opened in Bloomsbury in 1929.

Emphasis was on research and public health; the Seamen's Hospital Society backed out and the hospital was left high and dry. This marked a sad day for clinical tropical medicine in London, because basic research needs a strong clinical input. The hospital continued to move, ending up in its present home in St Pancras Hospital in 1951.

Gordon Cook is a clinician who has served in the Nigerian Army, has been a professor of medicine in Zambia, Riyadh and Papua New Guinea, and is now a consultant at the Hospital for Tropical Diseases in London. He has burrowed deeply into the contemporary documents and his story of triumphs and disasters,



Keeping illness at bay — Greenwich Hospital and the *Dreadnought*, 1841. (From *From the Greenwich Hulks to Old St Pancras*.)

been at sea since boyhood and were quite unable to cope with life ashore. They mistrusted everything, including the hospitals, which they refused to enter when they fell ill.

Wilberforce and other philanthropists drew attention to the problem and in 1823 the Seamen's Hospital Society was founded. The Navy lent the society the *Grampus*, an old 48-gun stores ship; it was moored at Greenwich and refitted as a hospital, and it accepted, without paperwork or bureaucracy, sick seamen of all nationalities from ships entering the Port of London. It was an immediate success and before long was replaced by the *Dreadnought* and other, larger vessels. The Greenwich hospital ships served for half a century and admitted more than 100,000 sailors.

But the ships were draughty, unhygienic and inconvenient, and in 1870, after some argument, the Navy leased part of the Greenwich Hospital to the Seamen's Hospital Society. That part was still called the *Dreadnought* and its activities were soon greatly expanded, with a branch hospital opening at the Victoria and Albert Dock. Many of the

who discovered the role of the mosquito as the intermediate host of the filarial worm that causes elephantiasis, and whose advice and support later helped Ronald Ross to elucidate the mosquito transmission of malaria. In 1890 Manson was back from the Far East, lecturing in London and arguing that Britain, as the hub of a great tropical empire, needed to teach tropical medicine as a new discipline, and needed schools to teach it in. The idea was supported by Joseph Chamberlain, the colourful Secretary of State for the Colonies, and a new wing was built, with government support, at the Victoria and Albert Dock Hospital to house a new London School of Tropical Medicine. Controversy broke out — the physicians at the *Dreadnought* were jealous and there was a long and acrimonious correspondence in the medical press — but the school was founded in 1899 and remained there until disrupted by war in 1914. It gained a high reputation, and by 1920 had moved to central London, where the Rockefeller Foundation was already offering assistance to build a School of Hygiene. The institutions were com-

achievements, rivalries and missed opportunities is allowed to speak for itself through the inclusion of letters, official reports and commentaries of the time, enlivened by *Punch* cartoons and photographs of the places and people concerned. This is a longer and more detailed account, and the style more restrained, than that of the ebullient Sir Philip Manson Bahr (Manson's son-in-law) who wrote a history of the school, full of biographical detail and personal reminiscences, in 1956. Cook resents the present neglect of clinical tropical medicine in Britain and the continued failure to renew an effective link between the school and the hospital. With the present uncertainty over the fate of teaching hospitals in London, the flagship of tropical medicine could be sunk and lost for ever and "Manson's achievement (like that of the British Empire) will become a mere memory of a bygone era". □

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Satellites in decay

David W. Hughes

A Tapestry of Orbits. By Desmond King-Hele. Cambridge University Press: 1992. Pp. 244. £35, \$54.95.

BEING in the right place at the right time is of considerable importance when it comes to having a successful scientific career. Desmond King-Hele managed all three. The place was the Royal Aircraft Establishment (RAE) at Farnborough, England, the heart of British aerospace and guided-weapons research. He started work there in the early 1950s immediately after completing a mathematics degree at the University of Cambridge; and he stayed there until he retired in 1988.

Among his early tasks was a design study of the Skylark rocket and the British reconnaissance satellite. The latter work led to the investigation of the effects of both air drag and the non-symmetry of the Earth's gravitational field on the orbits and return paths of low-altitude satellites.

The right time was the dawn of the space age. The launch of Sputnik 1 on 4 October 1957 saw scientists such as King-Hele swept out of their academic 'ivory tower' into the maelstrom of real-life engineering. Theoretically, he had shown how the addition of only a small upper-stage could convert a ballistic rocket into a satellite launcher. Fortunately, the upper-stages of the Soviet Union's rockets were huge: Sputnik 1's was 20 metres long and Sputnik 2's measured 31.8 by 2.95 metres. The launch system was such that both upper-stages stayed in orbit after releasing their satellites and reflected sufficient sunlight that they appeared brighter than the Pole Star in the night sky. People in their thousands came out to watch them as they crossed the sky every hour-and-a-half. They were so bright that their paths could be easily measured. King-Hele's theories could be verified and it soon became clear that they were correct and that the orbits were decaying quickly. The orbital period decreased by about 2.3 seconds per day, indicating that the atmospheric density near perigee (250 kilometres above the Earth's surface) was about eight times what was expected.

If the launch had been a year earlier, King-Hele's theories would not have been ready. If the first Sputnik had appeared a year later he would not have had a 'flying start'. Also, a new digital computer (called Pegasus) had just been installed at RAE. This enabled the orbit

and ephemeris to be calculated in a few days as opposed to a few months. And coupled with these advantages was the unintentional assistance of the then Astronomer Royal. Richard Wooley did not want the Royal Greenwich Observatory to have anything to do with space research and he insisted that all satellite observations were sent to RAE as quickly as possible.

In *A Tapestry of Orbits*, King-Hele skilfully entwines a thorough, well illustrated and well referenced review of his scientific speciality with revealing snippets of autobiography. The mixture works very well. We read of how discoveries came thick and fast. Not only was the density of the upper atmosphere found to be greater than extrapolations indicated, but it also seemed to vary unpredictably from day to day, between day and night and as a function of the phase of the solar activity cycle. It was found to be windy up there, the upper atmosphere rotating some 25 per cent faster than the Earth's surface. Also the orbits of the satellite were very sensitive to small changes in the gravitational field. A 40 metre deformation of the Earth's surface leads (depending on orbital inclination) to a 5 to 10 kilometre oscillation in the height of a satellite's perigee. The even terms in the multipolar expansion of the Earth's potential energy field could be obtained from the rate of rotation of the satellite's orbital plane and the odd terms came from the rate of change of eccentricity. Ground-based geodesists quickly saw their two centuries of laborious measurements of the Earth's shape completely upstaged by upstart space scientists.

King-Hele was helped by a host of amateur satellite spotters who provided accurate positional data, and he did an enormous amount of work to encourage these people by providing predictions of satellite positions. The media were helpful too. Satellites were burning out in the atmosphere at the rate of one every fortnight, and every time interest seemed to flag, a large satellite such as Cosmos 954 or Skylab enlivened the subject by scattering bits of metal over northern Canada and Australia. Friendly competition between King-Hele's group at RAE and the group at the Smithsonian Astrophysical Observatory also provided a spur. *Nature* published most of the results, and King-Hele fondly looks back on those happier days when "fortunately the refereeing system had not reached its present level of oppressiveness". With *A Tapestry of Orbits*, he has presented us with a first-class cocktail of science and life. □

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Bones of contention

S. M. MacLaughlin-Black

Bones: A Forensic Detective's Casebook. By Douglas Ubelaker and Henry Scammell. Edward Burlingame Books (Harper Collins): 1992. Pp. 317. \$23.

THIS book presents a rapid-fire succession of cases in forensic osteology. They are recounted by Dr Douglas Ubelaker, a curator of anthropology in the National Museum of Natural History at the Smithsonian Institution in Washington. He is undoubtedly an experienced, conscientious, meticulous and busy scientist, but as an author he leaves one feeling unfulfilled and shell-shocked by the constant bombardment of often apparently unrelated and irrelevant cases.

Perhaps the co-writer is to blame — all we know about him is that he's an author. Yet the colloquial literary style, described on the dust jacket as having the "frisson of a thriller", falls far short of the ability to combine and capture our fascination with crime, detectives and murder. As such, the book's intended audience is not obvious.

But there are some light moments. On one occasion, Ubelaker is found trying to duplicate marks on a bone in an attempt to identify the implement used in a murder. He purchases some chicken wings, which he proceeds to slam in a car door and run over with a lawnmower. Truly the committed scientist.

The text contains several factual mistakes. For example, it has been known for some time that pits on the dorsal surface of the pubis cannot be considered evidence of parturition, and I was dismayed to find in the diagram of the skeleton that the authors think we have only five cervical vertebrae.

One particularly disturbing incident is reported with some obvious satisfaction. After having positively identified a body, Ubelaker was contacted by the victim's mother requesting confirmation from him that the victim was indeed her son, as the police had told her nothing. Without clearance from the investigative authorities, he took it upon himself to confirm the facts to her over the telephone. This would be gross professional misconduct in the United Kingdom.

Few books have been written on forensic osteology. Unfortunately, this one will do little to enhance the public image of the field and fails to capture its essence. □

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Leaping larvae, jumping genes

Jack Cohen

Larvae and Evolution: Toward a New Zoology. By Donald L. Williamson. Chapman and Hall: 1992. Pp. 223. £29.95, \$39.95.

WILLIAMSON has a theory about DNA transfer that he believes to be consonant with modern biology, but which I believe is contradictory to it. Marine larvae, to which his theory applies and on which he's an expert, have difficult names and affiliations, so imagine a farm parallel. What he has found is that labradors have terrier puppies, which grow up into labradors; and that horses have foals and that camels have foal-like babies. But from this he concludes that there has somehow been adoption, exchange and copying of DNA. Most biologists would disagree.

For example, the dromioid crabs (including the sponge crab *Dromia*) do not have larvae like those of other true crabs, but like those of the (anomuran) hermit crabs; the zoeae of the inachid crab *Dorhynchus* look like other inachid larvae, but have an array of points and spines exactly like those of *Homolus*, in another superfamily. The diversity of larvae among the echinoderm classes nearly parallels that of the adults, but a few species have no planktonic larval forms and very different development. If none of them had planktonic larval forms — and indeed if marine worms, molluscs and flatworms didn't — we would classify them differently and it would make a lot more sense, conforming to our so-called 'basic ideas' of protostomes versus deuterostomes, enterocoely versus schizocoely, primitive versus advanced: that is, the basic theories of 1920s zoology.

Williamson wants to 'rescue' these classical concepts, but at the enormous expense of the radical belief that different forms catch larvae from each other: he believes that the larval DNA programme probably crossed between genera, superfamilies and even phyla, mostly as a result of fertilization by foreign sperms. The receiving organism occasionally had compatible development, expressing what he calls the "paternal programme" before that of the "maternal" species and showing dissonance between larval and adult (supposed) phylogeny. His frontispiece, a respectable pluteus, purports to be from a sea squirt (*Ascidia*) egg fertilized by an *Echinus* sperm. But all other cross-fertilizations that have been described show *maternal* early development; they

may show some paternal characters *after* the phylotypic stage when zygote-prescribed messenger RNA begins to be transcribed. Pluteus stage is post-phylotypic, but not by much.

My difficulty with Williamson's hypothesis is this. If the swapping of DNA programmes explains the phylogenetic puzzles of dissonance between some larvae and their adult forms, then one must assume that such programmes are descriptive rather than prescriptive. But these larvae are so similar in the critical characters. If they were a bit different, we could indeed imagine that the same DNA programme, in a different setting, prescribed them. But for us to explain their being exactly alike, DNA must describe final products, which it (mostly) doesn't: DNA is not a description of what you finally get. I would much sooner appeal to atavism and/or to stringent selection of a precise structure for explanation of similar lar-

vae with disparate adults. Once we have to suppose that there is such fine tuning by selection, of course, all the problems can be explained as evolutionary convergence, without any DNA swapping at all. Because DNA is prescriptive, Williamson's idea itself requires such convergent fine tuning; and that renders his radical hypothesis unnecessary.

There are dissonances in lots of other assumed phylogenies. Most biologists would agree that metazoan developmental programmes are very subtle and that we don't understand much about them. So it is not surprising that there are many oddities that don't fit our prejudices. But we must not invent radical theories to hide our puzzlement or save these prejudices, particularly if it means hastily abandoning most of modern developmental biology. □

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Inordinate fondness for insects

E. A. Jarzembowski

Treatise on Invertebrate Paleontology, Part R, Arthropoda 4, Volumes 3 and 4: Hexapoda. By F. M. Carpenter. Edited by R. L. Kaesler. Geological Society of America/University of Kansas: 1992. Pp. 677. \$87.50.

It is widely known that living insects greatly outnumber the rest of the animal kingdom. But their geological history is a mystery to many entomologists and palaeontologists. William Hennig pointed the way forward by promoting amber studies and by critically discussing fossil taxa that he considered particularly relevant to phylogenetic reconstruction. In the same year as his *Die Stammesgeschichte* (1969), an introduction to hexapods (insects in the broad sense) appeared in the *Treatise on Invertebrate Paleontology*, a respected monographic serial seeking to "present a comprehensive and authoritative, yet compact, statement of knowledge concerning groups of invertebrate fossils". This followed earlier discussion by Frank M. Carpenter, Raymond C. Moore and Ernst Mayr that an insect *Treatise* would be of great benefit: the promised volume (now doubled in size) has finally arrived.

It is a truly remarkable achievement that a single author has concisely diagnosed and checked the type species of more than 5,000 genera, of which nearly 1,200 are illustrated. These taxa are distributed in no fewer than 38 orders (10 extinct), spanning some 390 million years from the lower Devonian to the Holocene (the past 10,000 years). The

bibliography runs to more than 2,400 references. Only at one point does the author's energy flag — in the taxonomically difficult order Blattodea (cockroaches). Stratigraphical ranges are not taken to stages of geological time, but this highlights the unresolved problems of international nonmarine correlation.

The main (acknowledged) drawback is that the literature cut-off date is the end of 1983. So only 777 families are included, although the total identified exceeded 1,080 in 1991 (*Fossil Record 2* edited by M. Benton, Chapman and Hall, in the press). Also, the phylogeny draws inevitably on Niels Kristensen's earlier (1981) review. A generic supplement is already a serious consideration; integrating the systematics of extinct and extant insects in a unified phylogeny will take a little longer, however.

Since its inception, the *Treatise* has been an essential work for all scientific institutions with an interest in the fossil record and evolutionary biology. After a long and successful career at Harvard University (his research students included E. O. Wilson), Carpenter has produced what is undoubtedly his *magnus opus* in his ninety-first year. What's more, it's a database of past insect biodiversity that will service biology and geology into the twenty-first century — when more insects than ever might join the fossil record. □

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Materials with structural hierarchy

Roderic Lakes

Many natural and man-made materials exhibit structure on more than one length scale; in some materials, the structural elements themselves have structure. This structural hierarchy can play a large part in determining the bulk material properties. Understanding the effects of hierarchical structure can guide the synthesis of new materials with physical properties that are tailored for specific applications.

HIERARCHICAL solids contain structural elements which themselves have structure. The hierarchical order of a structure or a material may be defined as the number (n) of levels of scale with recognized structure. For $n=0$, the material is viewed as a continuum for the purpose of analysis of physical properties; $n=1$ (first-order) could represent a latticework of continuous ribs or the atomic lattice of a crystal. Hierarchical structure can arise in natural and in man-made materials. In the latter the structural hierarchy may be intentional or unintentional. The simplest conceptualization of hierarchical structure is descriptive: to recognize that structural features occur on different size scales. At the next level of sophistication, the idea of hierarchical structure can be used in analysis to determine physical properties of the material or the structure. At each level of the structural hierarchy, one may model the material as a continuum for the purpose of analysis, although strictly speaking such an assumption is warranted only if the structure size at each level of the hierarchy is very different. Finally, the idea of hierarchical structure can be the basis for synthesizing new microstructures which give rise to enhanced or useful physical properties. Benefits can include improved strength and toughness, or unusual physical properties such as a negative Poisson's ratio. These structures are considered to be fractal-like¹, but they are not true fractals as n remains finite and the solid volume fraction does not go to zero even for large n .

The idea of macroscopic hierarchical frameworks can be traced back at least to Eiffel's design for his tower² (Fig. 1) and to bridges such as the Garabit viaduct. The Eiffel tower is third-order, and has a relative density ρ/ρ_0 (density ρ as mass per unit volume of the structure divided by density ρ_0 of material of which it is made) just 1.2×10^{-3} times that of iron² (which is weaker than structural steel). The rationale for the use of small girders in such a large structure was attributed to ease of construction³, although it had also been suggested by Mandelbrot¹ that Eiffel perceived a structural advantage. For comparison, the World Trade Center (New York) and the Pompidou Centre (Paris), both first-order, contain a volume fraction of structural steel⁴ $\rho/\rho_0 = 5.7 \times 10^{-3}$. The World Trade Center contains steel with a yield strain ϵ_y of 0.0033, 2–3 times as strong as 'mild' structural steel.

A more recent example is a proposal by Dyson⁵ to construct hierarchical frameworks in outer space. Dyson presented scaling arguments to the effect that very large structures could be constructed with low mass. Stress analysis of elastic buckling in hierarchical truss structures was to come later^{6,7}. In modern structural engineering, however, the tendency seems to be away from hierarchical structures; although these contain less material to achieve a desired strength, the costs associated with fabrication and maintenance currently exceed any saving in material cost.

Dense hierarchical materials

Composites and polycrystals. Practical fibrous composites commonly have a low order of hierarchical structure in which fibres are embedded in a matrix to form an anisotropic sheet or lamina; such laminae are bonded together to form a laminate (Fig. 2a).

In the analysis of fibrous^{8–10} composites, the fibres and matrix are regarded as continuous media when one is analysing the lamina; the laminae are then regarded as continuous in the analysis of the laminate. The stacking sequence of laminae and the orientation of fibres within them governs the anisotropy of the composite. A similar continuum assumption is used in the analysis of particulate composites¹¹ and of foams¹². Inorganic crystalline materials have a 'hierarchy' of structural features¹³ such as grain boundaries between crystals of sizes ranging from millimetres down to micrometres, dislocations, and point defects such as vacancies on the atomic scale. These structural features give rise to viscoelastic behaviour^{14,15} manifested as attenuation of stress waves or damping of vibration at different frequencies. Polycrystalline materials can now be synthesized with a distribution of grain sizes less than $1 \mu\text{m}$ (nanocrystalline materials)^{16–19}. The small grain size, and hence large interface area, gives rise to desirable properties such as superplasticity (in which large irreversible deformation can occur without fracture), and improved strength and toughness. Small grain size also implies short diffusion distances, allowing processes that depend on diffusion, such as sintering, to occur at lower temperatures than would otherwise be possible.

Hierarchical laminate structures have been analysed theoretically to approximate the stiffness of polycrystalline aggregates²⁰, and for exploring bounds on the electrical conductivity of poly-

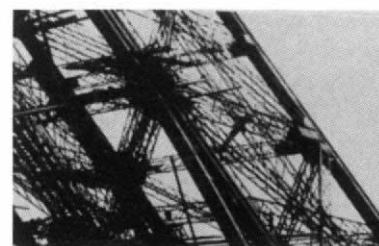


FIG. 1 Hierarchical structure in the Eiffel tower, after Loyrette². Inset: detail of leg.

crystals^{21,22} and the elastic stiffness of polycrystals²³ and composites²⁴. In these laminates, each lamina is composed of further laminae (Fig. 2b). For elastically isotropic hierarchical laminates, it is possible to attain²⁴ the theoretical upper or lower bounds²⁵ on the stiffness. These laminates are considered to be a mathematical tool rather than practical composites because widely differing length scales must be chosen to justify the assumption that each level is a continuum²³. Negative Poisson's ratios, which imply that the material becomes fatter in cross-section when stretched, are predicted in hierarchical laminates²⁶ with a chevron structure (Fig. 3a). The physical mechanism for the unusual Poisson effect is illustrated by the hinged framework which unfolds under tension (Fig. 3b). One can achieve, with these laminates, Poisson's ratio values approaching the lower limit of -1 for mechanically isotropic materials.

Polymers. Polymers can exhibit structural hierarchy on the molecular, ultrastructural and microstructural levels²⁷. In crystalline polymers, there are spherulites on the scale of tens of micrometres, the spherulites themselves contain a lamellar texture and the molecules within the lamellae contain structure. Amorphous polymers have structure on the molecular scale only²⁷. When they are irreversibly deformed, however, crazing occurs and the process can be understood with the aid of a hierarchical approach which can deal with the multiple size scales involved. Crazes are bridged by nanoscale microfibrils whose properties are important. At the macroscale the crazed material can be considered as a composite. In covalent amorphous solids, the concept of hierarchical order has been used to aid the classification of order²⁸ into short-range (2–5 Å), medium-range (5–20 Å) and long-range (≥ 20 Å).

Biological materials. Human compact bone is a natural composite which exhibits a rich hierarchical structure^{29,30} (Fig. 4). On the microstructural level are the osteons³¹, which are large (200 μm diameter) hollow fibres composed of concentric lamellae and of pores. The lamellae are built of fibres, and the fibres contain fibrils. At the ultrastructural level (nanoscale) the fibres are a composite of the mineral hydroxyapatite and the protein collagen. These specific structural features have been associated with various physical properties. For example, the stiffness³² of bone arises from the composite structure of mineral microcrystals and protein (principally collagen) fibres. Slow creep³³ results from slip at cement lines between osteons. The cement lines as weak interfaces impart a degree of toughness³⁴ to bone. As for pores, the lacunae are ellipsoidal pores which provide space for the osteocytes, the living cells of bone. The bone cells at this

level of scale permit bone tissue to remodel its structure in response to prevailing stresses³⁰. Haversian canals are cylindrical pores containing blood vessels which nourish the tissue. Canaliculi are fine channels radiating from the lacunae. Mechanical stress due to physical activity is considered to be important in pumping nutrients through these channels³⁵. The pore structure of bone is essential in maintaining its viability and consequently its ability to adapt to mechanical stress. A two-level hierarchical analytical model³⁶ has been used to predict its anisotropic elasticity; it successfully modelled how bone stiffness depends on the orientation of applied stress with respect to the osteon axis.

Other examples of natural hierarchical materials include wood^{37,38}, tendon²⁷, trabecular (spongy) bone and bamboo. Of these, only tendon may be regarded as 'dense'; the others are cellular. Tendon consists of collagen which on a molecular scale is similar to that of bone²⁷. The triple helical collagen macromolecule is formed as a result of the amino acid glycine occupying every third unit. The strongest intermolecular attractions occur when neighbouring molecules are shifted by 67 nm, the 'stagger' which is responsible for the banded appearance of collagen observed by electron microscopy. Assembly of subfibrils into fibrils is thought to be controlled at least in part by the primary structure of collagen. In tendon, the collagen forms fibres which are organized into mostly parallel fibre bundles of progressively larger size. The larger-scale organization is attributed to interaction with noncollagenous components such as proteoglycan matrix. The fibres are not perfectly aligned; they form a wavy or crimped structure which confers on the tendon an initial compliance as the fibres straighten under load. The damage processes that governs the strength and toughness of tendon involve structural elements over the full hierarchical range of sizes.

Role of the largest structural elements

Structure may be present on many size scales, but the largest structural elements often have a unique role. If the largest structure is not negligible in size compared with the object itself or a crack or hole in the object, the classical continuum view may no longer describe the situation adequately. When deformed elastically, objects with large structural elements may exhibit size effects in bending and torsion³⁹: 'classically', the rigidity of rods in bending or torsion should be proportional to the fourth power of the diameter, but slender rods can be stiffer than this. Moreover the stress concentration predicted in classical elastic solids near holes and notches is alleviated in some materials with microstructure³⁹. This is beneficial in structural materials. In some foams, incomplete cells near a cut surface contribute to the volume but not to the stiffness or strength⁴⁰ so that small objects are less stiff than expected from classical continuum analysis (the converse of the slender rods). In hierarchical composites, the largest structural elements such as fibres^{41,42} or particulate heterogeneities⁴³ seem to govern the fracture toughness and the localization of microdamage. Classical elasticity theory has no length scale associated with it. More general continuum theories such as Cosserat (micropolar) elasticity^{44,45} allow rotation of points in the continuum as well as translation, and contain characteristic lengths as well as stiffnesses among the material constants. Physically the additional freedom in the continuum corresponds to twisting or bending motions in the fibres or ribs in the material microstructure. Generalized continuum theories offer predictive power in dealing with nonclassical phenomena^{39–43}, and may be of use in future analysis of hierarchical materials in which one relaxes the assumption that the structure size at each hierarchical level is very different.

Hierarchical cellular materials

Cellular solids. Cellular solids are composites in which one phase is solid and the other is empty space, or possibly a fluid. Natural

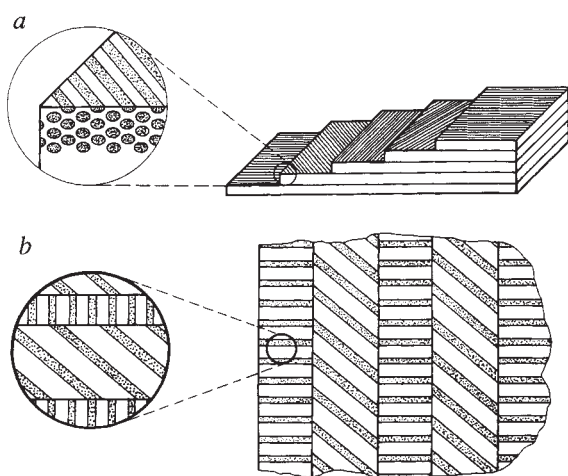


FIG. 2 *a*, Hierarchical structure of order two in a practical fibrous laminate. Parallel fibres form a lamina; laminae of different orientations are stacked to form a laminate with specified anisotropy. *b*, Hierarchical laminate, adapted from Milton²³; order three shown. Each lamina contains a sublaminate. These laminates are used in mathematical demonstrations of attainable material properties in composites.

examples are rocks, wood and bone. Porous rocks can have a wide range of pore sizes⁴⁶, but the structure is not as highly organized as that of wood and bone. Wood contains elongated pores (called tracheids or fibres) oriented along the tree or limb axis, radial channels called rays, and larger sap channels^{12,47}. The cell walls are themselves fibrous and consist of oriented cellulose in a hemicellulose and lignin matrix. The alignment of the tracheids is favourable for resisting the prevailing forces in the tree¹². Trabecular bone has a spongy structure. The struts or ribs in trabecular bone have a complex internal structure (Fig. 4) similar to that of the osteon in compact bone.

Synthetic cellular materials are used for applications such as cushioning, filtration, insulation and lightweight sandwich cores. They tend to have either a two-dimensional or a three-dimensional structure (honeycombs and foams respectively). In most synthetic cellular solids, there is only one size scale other than the atomic: that of the cells. Some synthetic open-cell polymer foams can have an unintentional further hierarchical structure, however, in that the ribs may contain 'microcells'⁴⁸. Aerogels are gels in which the fluid phase is air rather than solvent; they have submicroscopic pores with a wide range of sizes organized in a hierarchical structure^{49,50}. The microstructure depends on density, and the Young's modulus varies as $\rho^{3.8}$. Foams with negative Poisson's ratio⁵¹ have an inverted (re-entrant) cell shape in which the cell ribs bulge inward rather than outward. Although they are not hierarchical in themselves, they may be used to make hierarchical composites in which the open space in the cells is filled with a compliant solid or with a foam of smaller cell size. If the filler is viscoelastic, the composite's viscoelastic response can be made large when the filler experiences a larger local strain than does the composite. By choosing the relaxation rates of the large-cell foam and the filler, it is possible to design materials in which Poisson's ratio increases or decreases⁵² with time.

The first quantitative analysis of open hierarchical structures is that of Parkhouse⁶, who even proposed a competition for the most structurally efficient hierarchical design. Parkhouse con-

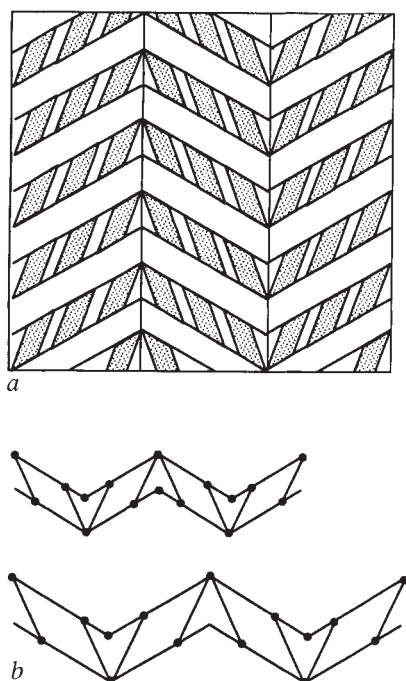


FIG. 3 a, Negative Poisson's ratio laminate of Milton²⁶. b, Rod-and-hinge frame structure to illustrate the mechanics of the laminate in achieving a negative Poisson's ratio.

TABLE 1 Parameters in equations (1) and (3)

Microstructure	Stiffness		Strength	
	Eq. (1) Exponent r	Eqs. (1, 3) Multiplier k	Eq. (3) Exponent q	Eq. (3) Multiplier a
Foam, open cell, ribs bend ¹²	2	1		
Elastic buckling		1	2	0.05
Plastic buckling		1	3/2	$0.3\epsilon_{y, \text{solid}}$
Honeycomb (out of plane) ¹²				
(Cell walls compress axially)	1	1		
Elastic buckling		1	3	5.2
Plastic buckling		1	5/3	$5.6\epsilon_{y, \text{solid}}$
Brittle crushing		1	1	$12\epsilon_{\text{ult, solid}}$
Ductile crushing		1	1	$\epsilon_{y, \text{solid}}$
Frame (ribs deform axially)				
Elastic buckling				
oriented	1	1	2	0.05
cubic ⁹	1	1/3	2	0.05
isotropic ⁹	1	1/6	2	0.05

sidered lattice, truss, honeycomb and tubular geometries, and showed that hierarchical structure can be used in the design of structural elements which, for a given compressive strength, are much lighter than elements with simple structure. The effect of damage on reliability in homogeneous solids, in structures with one scale size and in those with hierarchical structure were described⁵³ in connection with continuum models of structures. Ashby⁵⁴ has elucidated the freedom that a designer of load-bearing components has in choosing material properties, section shape, and with cellular and composite materials, microstructural degrees of freedom.

Prediction of strength and stiffness. In cellular materials, the stiffness depends on density and on the structure. In many such materials, which ordinarily have structure only on the scale of the cells, the relationships are simple. The Young's modulus (stiffness) E of a cellular material such as a foam or honeycomb (considered as a continuum) is given in terms of the Young's modulus E_0 of the solid from which the material is made, the density ρ_0 of the solid phase and the density ρ of the foam¹². The atomic structure is ignored here because it is absorbed in the continuum description of the cell ribs.

$$E/E_0 = k[\rho/\rho_0]^r \quad (1)$$

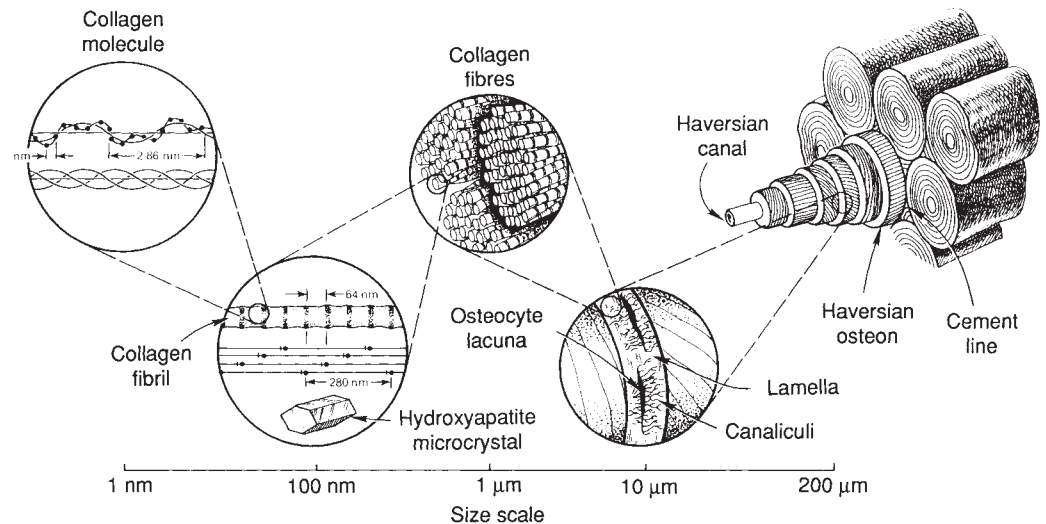
The values of k and r depend on the type of structure (Table 1). In this section, stiffness and strength in hierarchical cellular solids is predicted for 'conventional' (not re-entrant) materials: honeycombs with hexagonal cells and for open-cell foams with convex (usually tetrakaidecahedral) cells. Simple continuum models now available for cellular solids¹² aid the analysis. Hierarchical cellular solids are considered in which the material making up the cell ribs is also cellular and has a smaller cell size. For a solid material viewed as a continuum, the hierarchical order $n=0$; for a conventional foam or honeycomb, $n=1$; and for a sponge with porous ribs, $n=2$. Hierarchical solids may be envisaged with any hierarchical order, with an upper bound on n determined by the fact that the smallest cells must be of larger size than atomic dimensions.

To predict the properties of hierarchical cellular solids, one can iterate the stiffness equation (1), considering the solid density to be ρ_0 at zeroth order. Strictly, the classical continuum view used here is valid only if the size of the structure making up each cell wall or rib is much smaller than the rib itself.

$$E_n/E_0 = k^n[\rho/\rho_0]^r \quad (2)$$

On this basis, we see that for honeycombs deformed out of plane and for foams ($k=1$), the hierarchical order n does not influence the stiffness, whereas for framework-type structures for which $k < 1$ the stiffness decreases with n . The relationships

FIG. 4 Hierarchical structure in human compact bone; individual size scales adapted from refs 28–31. Fibrous, laminar, particulate and porous structure is present at different size scales.



for compressive strength¹² σ , or maximum stress before collapse, of conventional first-order materials ($n = 1$ for cellular material, $n = 0$ for the solid phase) can be rewritten in a general form for two arbitrary hierarchical orders differing by one, with $n \geq 1$. Here a and q are parameters which depend on the rib failure mode and material structure (see Table 1).

$$\sigma_n = akE_{n-1}[\rho_n/\rho_{n-1}]^q \quad (3)$$

Possible failure mechanisms are elastic buckling of cell ribs in which the ribs reversibly collapse, plastic buckling in which the ribs irreversibly collapse, or crushing in which the ribs fracture. As shown in Table 1, the strength parameter a for plastic buckling depends on $\varepsilon_{y,solid}$, the strain at yield for the solid from which the material is made; for crushing, it depends on $\varepsilon_{ult,solid}$, the fracture strain of the solid. After some manipulation, and assuming the density ratio is the same for each level, we find that the strength to density ratio of the hierarchical material is, for $n \geq 1$

$$\frac{\sigma_n}{\rho_n} = \frac{E_0}{\rho_0} ak^n \left(\frac{\rho_n}{\rho_0} \right)^{r-1+(q-r)/n} \quad (4)$$

The upper bound on the strength-to-density ratio is that of the solid at the zeroth level of the hierarchy. The crush limit for honeycomb is independent of hierarchical order: as $k = 1$ and $q = r = 1$, n has no effect.

The predicted strength-to-density ratio of honeycombs is shown in Fig. 5. The physical mechanism for the improved strength is the suppression of buckling in the hierarchical structure. Because failure can occur by elastic buckling, plastic buckling or crushing, the actual strength corresponds to the lowest stress of the possible failure modes. For low-density honeycombs, marked improvement in compressive strength can be realized in hierarchical structures. Most of the gain in strength occurs in the first few levels of the hierarchy; the situation for large n is one of diminishing returns. Moreover, the relative thickness of the cell walls increases with n , so for a relative density of 0.01, values of n above 4 are unrealistic. If the solid phase had a higher ultimate strain (for example $\varepsilon_{ult} = 0.054$ for glass fibres; 0.08 for silica) the transition from elastic buckling to plastic buckling in Fig. 5 would occur at higher stress. Consequently, there is a considerable advantage to using high-strength materials in making a hierarchical honeycomb, in contrast to conventional honeycomb in which only the stiffness of the solid phase is important.

As for foams, conventional open-cell foams deform by rib bending¹² for which $r = 2$ and $k = 1$ in equation (1). For such foams, there is no strength advantage associated with the hier-

archical structure (Fig. 6). The situation is different for an oriented foam^{12,55} such as trabecular bone of a particular structure (modelled as first-order) in which deformation proceeds by axial rib extension with $r = 1$ and $k = 1$ in equation (1). A cubic lattice of beams ($k = 1/3$), more representative of building construction than of foams, deforms axially in this way. Such materials and structures are predicted to have a considerable strength advantage associated with the hierarchical structure (Fig. 6). For large n , the cubic and isotropic microstructures perform less well, because at each level, some ribs are oriented so that they do not support load.

These predictions of stiffness and strength are independent of scale and are relevant to large structures as well. If elastic buckling were the only failure mode considered (as in ref. 6), the saving in strength or weight of these structures would be overestimated.

A different type of analysis which generates hierarchical structure is finite-element analysis in which the topology of a structural element is allowed to vary⁵⁶. In finite-element analysis, stress and deformation fields in an object are analysed by subdividing a model of the object into many small segments, for each of which it is simple to compute stress. In most applications

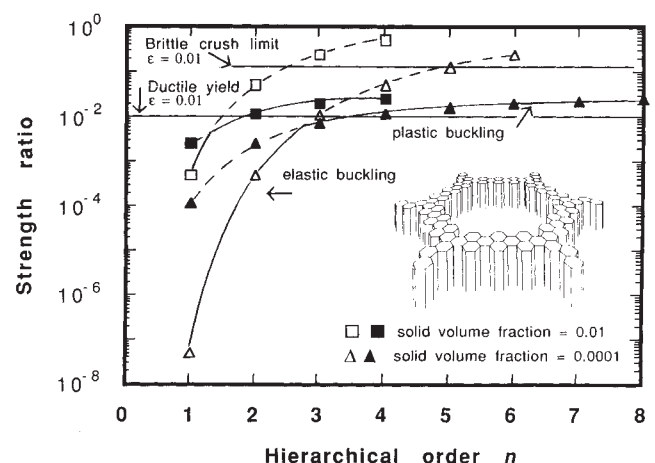


FIG. 5 Strength-to-density ratio of hierarchical honeycomb increases with hierarchical order n . Normalized strength ratio $(\sigma_n/\rho_n)/(E_0/\rho_0)$, for hierarchical honeycomb microstructure, as a function of n , for several solid volume fractions ρ_n/ρ_0 : squares, 0.01; triangles, 0.0001. For the solid, the yield strain and fracture strain are assumed to be 0.01. Open symbols: elastic buckling. Solid symbols: plastic buckling. Inset: second-order honeycomb cell. Solid curves: buckling mode with lowest stress limits the strength.

of the method, the boundaries of the model are not changed. In ref. 56, holes were deliberately introduced in the model, and their boundaries were progressively modified depending on the calculated stress at each iteration. Optimization of the structural element to maximize stiffness for given weight leads to a cellular microstructure which becomes truss-like if the solid volume fraction is small. The optimal microstructure under some conditions is hierarchical.

Second-order hierarchical honeycombs can easily be made by the techniques currently used to make practical expanded honeycomb. In the 'hobe' (honeycomb before expanding) block method, strips of material are bonded together with bands of adhesive so that the bonded regions of one strip lie above the unbonded regions of adjacent strips. To produce a honeycomb, the width of glued and unglued sections is made equal. The stack of strips is pulled apart so that the web between the bonded strips forms the cell walls. I have used a modified hobe-block approach to make hierarchical honeycomb by stacking unexpanded small cell layers and using wide glue strips between them to make large cells with small cells in the walls. I also constructed hierarchical honeycomb by cementing together segments of first-order honeycomb to form a larger honeycomb. Experiments using a servohydraulic test machine to crush the specimens showed that second-order paper honeycomb is a factor of 3.2 to 3.8 times stronger in compression than first-order honeycomb of the same density (0.01 g cm^{-3}). The simple theory developed above, assuming plastic buckling, predicts a strength enhancement factor of 4.6. Given the idealizations involved, including identical density ratio at each order, this agreement seems reasonable. Honeycomb made of a stronger material would be enhanced even more in strength by hierarchical structure, as the transition between the governing failure modes depends on the strength of the material used.

Conclusions and prospects

Many materials exhibit hierarchical structure; the hierarchical aspects of structure are useful for descriptive purposes, for analysis and for synthesis. Hierarchical cellular material microstructures sometimes have far higher compressive strength than cellular solids of similar density with conventional structure. Two-dimensional hierarchical cellular solids (honeycombs) are

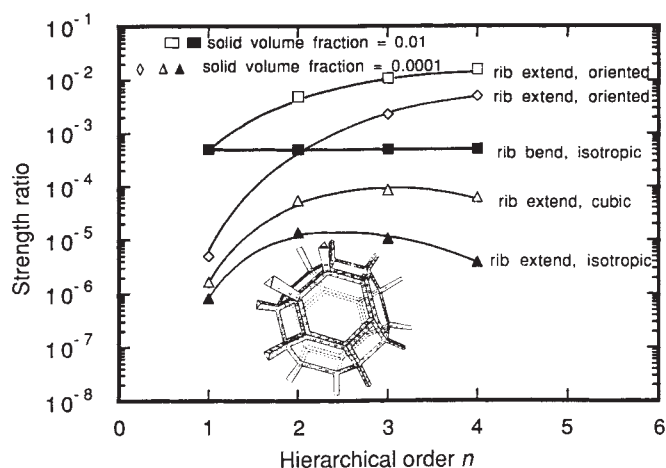


FIG. 6 Strength-to-density ratio of hierarchical foam can increase with hierarchical order n . Normalized strength ratio $(\sigma_n/\rho_n)/(E_0/\rho_0)$, for different types of hierarchical foam microstructure, as a function of n , for several volume fractions ρ_n/ρ_0 . Solid is assumed to be sufficiently strong that failure occurs by elastic buckling. Inset: second-order foam cell, showing rib bend.

easily made, and other hierarchical cellular solids could be manufactured by rapid prototyping systems⁵⁷ in which a computer-generated design is converted into complex shapes by photochemical, sintering, deposition, layering or sculpting techniques. Amongst the useful material properties that may be conferred by hierarchical structure, an intriguing possibility is that of simultaneously achieving values of strength and toughness, for which ordinarily there is a trade-off. Hierarchical structure can give extremal values of unusual properties such as negative Poisson's ratios. There is the further possibility of designing materials with extreme values of properties such as thermal expansion or piezoelectricity. □

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A supernova remnant associated with the young gamma-ray pulsar PSR1706–44

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THE Energetic Gamma Ray Experiment Telescope (EGRET) on the Compton Gamma Ray Observatory satellite recently detected¹ pulsed γ -radiation from the radio pulsar PSR1706–44; this is only the fourth radio pulsar to be identified as a γ -ray source. The other three (Vela, the Crab and PSR1509–58) are all associated with supernova remnants (SNRs), whereas very few—perhaps four—of the remaining 500 or so galactic radio pulsars have convincing associations with SNRs². We have mapped the field around PSR1706–44 at 843 MHz with a resolution of 44 arcsec using the Molonglo Observatory Synthesis Telescope, and have identified a shell-type SNR at a distance of about 3 kpc—consistent with the distance deduced for the pulsar. The pulsar is seen as a slightly variable point source, located in an enhanced knot on the arc of the SNR shell.

PSR1706–44 lies within the 1° radius error circle of the COS-B source 2CG342-02 at RA = 17 h 06 min 05.1 s, $\delta = 44^\circ 25' 0''$ (epoch 1950 coordinates are used throughout) and accounts for most, if not all, of the γ -ray flux from the area. Between August 1975 and December 1978 the COS-B satellite observed 2CG342-02 on five occasions and catalogued it as the tenth-strongest γ -ray source. The position of the centre of the error circle was given³ as $l = 342.9^\circ$, $b = -2.5^\circ$ (RA = 17 h 04 min 36 s, $\delta = -44^\circ 28'$).

In June 1984 we searched a $70' \times 100'$ field centred on 17 h 04 min 30 s, $-44^\circ 20'$ with the Molonglo Observatory Synthesis Telescope (MOST) array and in May 1986 observed two adjacent fields to complete a search within the error circle of the COS-B γ -source. This revealed the shell of a faint SNR seen over the western half of an ellipse having major and minor axes $\sim 44'$ and $32'$ respectively. Near the centre is the strongest source in the field at 17 h 04 min 51.79 s, $\delta = -44^\circ 14' 29.2''$, slightly extended with peak flux of 276 mJy and integrated flux 305 mJy. This may be a star-formation region and site of the supernova or just a positional coincidence. Several other point sources in the field range from 100 to <10 mJy. Most of these are presumably extragalactic but they include the pulsar PSR1706–44, although this was not recognized at the time. Following the publication of the survey⁴ containing details of the radio pulsar and the announcement⁵ that it emits γ -rays, we observed the region on a further three epochs in May, June and July 1992. Comparing these, the point sources have relative calibration errors of ± 0.06 s in RA, $\pm 0.5''$ in declination and ± 0.6 mJy in flux density. Figure 1 shows the MOST image of July 1992.

The SNR shell shows clearly on all images in the west but is too faint to follow to the eastern half. The arc is brightest to the NW at 17 h 03 min 22 s, $-44^\circ 06' 30''$, away from the pulsar but closest to the galactic plane. The pulsar lies at the southeast end of the SNR shell and is seen as a point source with a mean continuum flux of 20.7 mJy. It is significantly variable with its peak flux at the four epochs 22 June 1984, 25 May 1986, 8 May 1992 and 11 July 1992 being 23.2, 18.4, 19.8 and 21.5 mJy respectively. It may be confused by parts of the shell, for the mean position 17 h 06 min 05.27 s, $-44^\circ 25' 20.2''$ is displaced $5.5 \pm 1.1''$ at PA 160° from the pulsar timing position.

The integrated flux density for the shell in Fig. 1 is $3.28 \pm$

0.24 Jy. Even when the contribution of ~ 1200 mJy from all small-diameter sources in the field is included, the total seen by MOST at 843 MHz is only ~ 4.5 Jy, much less than observed by filled-aperture telescopes. Most Southern Hemisphere SNRs discovered so far have been identified using several Molonglo Cross (408 MHz) surveys, yet the galactic plane map⁶ for $|b| \leq 3^\circ$ shows only faint emission from the region of 2CG342-02. The published maps of the 5-GHz galactic plane survey⁷ do not cover the SNR, but an unpublished part (R. F. Haynes, personal communication) extends to a declination of $-44^\circ 10'$ and shows the northern edge of the shell. The 1960s radio surveys made with telescopes of beamwidth $\sim 50'$ detected strong radio emission at four frequencies from an unresolved source within the 1° radius COS-B error circle. The source designation and detected fluxes are: MSH17–41 (85 MHz) 25 ± 4 Jy, KOM43 (408 MHz) 45 Jy, CTB36 (960 MHz) 30 Jy, MHR59 (1,440 MHz) 15 ± 7 Jy. All sources lie within a beamwidth of each other, although the quoted positions are discrepant. None of the four surveys detected two separate sources from this region, so it seems that all the sources are the same extended object, and that this lies close to 17 h 03 min, $-44^\circ 20'$. The most recent observation of the region with a filled-aperture telescope is the $20'$ resolution survey⁸ made at 2.3 GHz: the map shown in Fig. 2 illustrates the relationship of the source to the SNR in the MOST map and the galactic ridge. The extended ($\sim 40' \times 25'$) source has a flux of 24 ± 7 Jy (J. L. Jonas, personal communication) and hence is nonthermal. The peak coincides with the brightest part of the SNR shell. It seems that there is considerable broadscale emission missing from the MOST map.

We now consider the estimates of distance to the SNR and the pulsar. One may use the surface-brightness–diameter (Σ – D) relation to estimate the physical diameter of the SNR and hence its distance. Although the use of such a relation has been

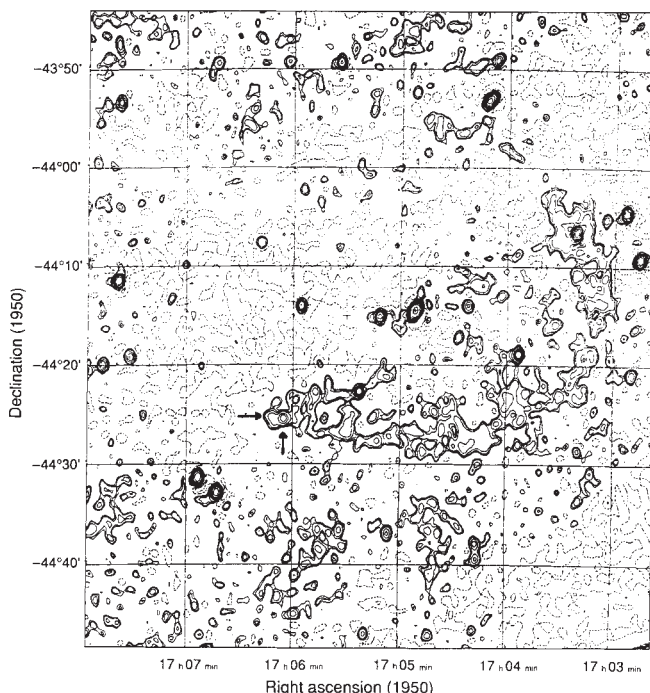


FIG. 1 MOST image at 843 MHz of the pulsar and supernova remnant 1706–44 observed 11 July 1992. The contour levels are: $-5, -2, 1.5, 2, 3, 5, 10, 15, 20, 30, 40, 60, 100, 200, 250$ mJy per beam of $44'' \times 63''$. The peak flux density in the field is 276 mJy for the extended source near the centre of the $44' \times 32'$ SNR half-ellipse arc. Each epoch has a relative gain calibration with r.m.s. error $\pm 0.9\%$; the 843-MHz flux density scale has a further absolute error of $\pm 8\%$. The r.m.s. noise at full resolution is 1.1 mJy per beam, and 0.5 mJy is smoothed to $2'' \times 2''$ for the faintest features. The position of PSR1706–44 is marked by arrows.

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queried⁹, the mean diameter for faint remnants, such as this, becomes insensitive to brightness. It is also fortunate that the uncertainty in the flux density for the SNR does not affect the brightness estimate significantly. MOST observes a hollow shell with a rim brightness typically 9 mJy per beam giving $\Sigma_{843} = 3 \times 10^{-21} \text{ W m}^{-2} \text{ Hz}^{-1} \text{ sr}^{-1}$. If we assume that there is an additional broadscale SNR emission filling the $44' \times 32'$ shell and take the 45 Jy of source KOM43 as the flux at 408 MHz, we have $\Sigma_{408} = 5 \times 10^{-21} \text{ W m}^{-2} \text{ Hz}^{-1} \text{ sr}^{-1}$. These are equivalent for a typical SNR spectral index of -0.5 .

There are a number of Σ - D relations to choose from. The relationship of Clark and Caswell¹⁰ gives $D = 38 \text{ pc}$. Caswell and Lerche¹¹ modified this relationship to include a z -dependence, which gives $D = 43 \text{ pc}$, whereas $D = 34 \text{ pc}$ is obtained from the expression of Allakhverdiyev *et al.*¹² which is restricted to classic shell-type remnants. The corresponding distances are 3.5, 4.0 and 3.2 kpc.

The quoted¹ distance of 1.5 kpc to the pulsar based on its dispersion measure of 75.68 pc cm^{-3} assumes that the mean interstellar electron density along the line of sight is $n_e = 0.05 \text{ cm}^{-3}$. This is larger than the frequently used value of $n_e = 0.03 \text{ cm}^{-3}$, which would put the pulsar at $\sim 2.5 \text{ kpc}$. A new model for the galactic electron density distribution (J. H. Taylor and J. M. Cordes, preprint) gives 1.8 kpc, placing the pulsar on the far side of the Sagittarius arm of the Galaxy. In the model this arm contributes more than half of the dispersion measure whereas most of the line of sight has a much lower electron density, $\sim 0.02 \text{ cm}^{-3}$. Because of the inhomogeneity of the interstellar medium there must be wide variations in the actual mean n_e across an arm along the line of sight to specific pulsars. For nearby ones such as this the uncertainty in the distance can be large. We believe that a distance of $\sim 3 \text{ kpc}$ as indicated for the SNR is consistent with the pulsar's dispersion measure.

We do not have 21-cm observations of this region with a sufficiently high resolution to look for evidence of a concentric outer neutral gas shell, but one can make use of an assumed uniform dust-gas ratio and examine the IRAS 100 μm sky plate covering this region. This shows no detailed correlation with the radio features, evidence that this is indeed a nonthermal rather than thermal source¹³. There is, however, clear evidence of a small ridge of higher 100- μm intensity protruding from below the galactic plane and curving around the southern side of the remnant. The length of the ridge is of the same order as

the size of the remnant. The intensity towards the centre of the remnant is slightly below that of its surroundings and this may represent a cavity with an outer perimeter shell of higher-density gas.

If the 100- μm ridge is associated with the remnant, the latter's distance could perhaps be better determined by the observation of the ridge at 21 cm. The Durham-Parkes survey¹⁴ has a resolution of $15'$ and covers this area. At the nearest sample points to the ridge ($l = 342.5^\circ, 343.0^\circ, 343.5^\circ; b = 2^\circ$), there is a velocity component at -32 km s^{-1} which is much less evident at adjacent sampled positions. The corresponding distances for this velocity from the galactic rotation curve are 3 and 14 kpc. A distance of 3 kpc would agree with the Σ - D estimates and place the remnant satisfactorily in the Norma spiral arm. This distance would also increase the estimate¹ of the required energy in γ -rays from 1.8% to 7% of the spin-down energy loss.

Caswell and Lerche¹¹ give an age-diameter relation based on the Sedov model. For a diameter of 34 pc this gives the SNR an age of 5,600 yr but the deduced age is dependent upon the assumed density of the medium into which the SNR is expanding.

The pulsar has a period of 102 ms and slows with a characteristic age of 17,300 yr. For the pulsar to move $18'$ from the apparent centre of the SNR in this time its proper motion would have to be $0.06'' \text{ yr}^{-1}$. Such motion should be detectable either from timing measurements, or from relative astrometry with the Parkes-Tidbinbilla Interferometer¹⁵ or with Parkes-Hobart very-long-baseline interferometry. There are two suitable reference sources southeast of the pulsar at RA = 17 h 06 min 42.89 s, $\delta = -44^\circ 32' 47.1''$ (88 mJy) and RA = 17 h 06 min 53.26 s, $\delta = -44^\circ 31' 18.8''$ (100 mJy).

If the distance is 3 kpc, the pulsar has moved 16 pc at a transverse velocity of 900 km s^{-1} . This approaches the highest transverse velocity so far directly measured (PSR2224+65) but is less than velocities of 1,700 and $2,300 \text{ km s}^{-1}$ inferred for the two young pulsars PSR1800-21 (W30; G8.7-0.1)¹⁶, and PSR1757-24 (G5.4-1.2)¹⁷, respectively. These are accepted already as associated with SNRs. These both have a characteristic age of 16,000 yr and have already reached or passed through the shell of their SNR: in so doing PSR1757-24 has apparently brightened the nearby shell by an injection of particles and magnetic field¹⁷. PSR1706-44, which has just reached the shell, seems not to have done this yet. These young SNRs will be in their adiabatic stage of development.

The high transverse velocities inferred for the three pulsars may be avoided by assuming that the pulsar and SNR are much older (by ~ 10 times) than the characteristic spin-down time¹⁷, so that the remnant is in the older radiative phase for which Shull *et al.*¹⁸ have discussed possible interactions.

X-ray emission from the pulsar has been detected by the Rosat satellite¹⁹, but we are not aware of any extended component detected from the SNR. Soft X-ray data obtained during an Exosat observation of the nearby galactic bulge source 2S1705-440 (J. Heise, personal communication) included a 9,000-s exposure with the imaging camera using a thin polypropylene filter. The 2.2° diameter field of view included the entire SNR. Two areas are of particular interest: the SNR shell, especially its brightest part, and the 305 mJy source near its geometric centre. There was no evidence of more than 2σ emission from the shell and no more than 1σ from the central source. We interpret this as giving an upper flux limit of 0.15 mCrab for these regions. Nevertheless it is likely that there is some extended emission associated with the young SNR which should be detectable by Rosat. $\square$

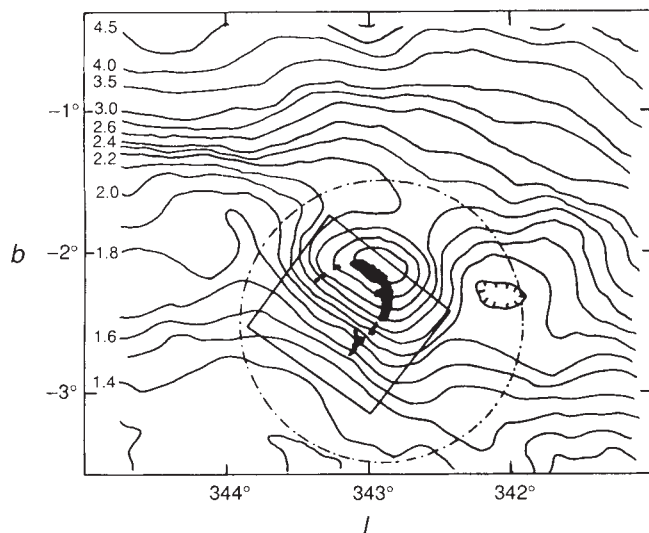


FIG. 2 Filled-aperture observation at 2,300 MHz (A. Greybe, personal communication) from the $20'$ resolution survey of Jonas *et al.*⁸ using the 26-m telescope at Hartebeesthoek, RSA. The COS-B error circle, the field of view of Fig. 1 and the outline of the SNR are superimposed.

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High-pressure viscosity of glycerol measured by centrifugal-force viscometry

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AS a liquid approaches its glass transition temperature T_g , its viscosity increases rapidly. The glass transition can be induced either by lowering the temperature through T_g or by increasing the pressure (and thereby the density) at constant temperature. The effect of temperature on viscosity is well studied, but the density dependence of viscosity close to T_g is less well understood. Here we report measurements of the viscosity of glycerol, one of the most widely studied glass-forming liquids, at pressures of up to 3 GPa using centrifugal-force viscometry in a diamond-anvil cell. We find that free-volume theory^{1,2}, which ascribes an incompressible hard-sphere volume to the molecules, provides a good description of the viscosity over the entire pressure range (and by extrapolation, up to the glass transition at ~ 5 GPa). We are thus able to predict the effect of pressure on T_g and on the glass fragility (the structural breakdown in the liquid close to the transition).

Glycerol has a glass transition temperature at atmospheric pressure of 187 K (ref. 3) Ultrasonic⁴, dielectric^{5–7} and specific heat⁸ measurements have been made at elevated pressures.

Viscosity has been measured over only two orders of magnitude to a pressure of 1.2 GPa (ref. 9).

We have shown previously that high-precision viscosity measurements to 10^6 cP can be made with the rolling-ball diamond-cell technique¹⁰. A capillary-type viscometer can be used for higher viscosities¹¹, but large (50%) errors and the fact that a single sample cannot be used over the entire viscosity range make its use undesirable. To allow measurements of higher viscosities, we have now developed a centrifugal-force viscometer (Fig. 1). This enables us to make high-pressure measurements extending over 10 orders of magnitude in viscosity on a single sample, while maintaining high precision.

Viscosity measurements were made on a sample of Aldrich spectrophotometric-grade glycerol (99.5 + %) which was dried over a molecular sieve. The ambient pressure viscosity of glycerol was determined in a Contraves Low Shear 30 Viscometer to be $1,130 \pm 30$ cP at 23 °C, indicating a water content of less than 0.2% (ref. 12). Figure 2 shows the viscosity measurements made by the high-pressure rolling ball and centrifugal force techniques at 22.5 ± 1.0 °C. These data extend over a pressure range of nearly 3 GPa and encompass a range of nearly seven orders of magnitude in viscosity, η . This exceeds the previously measured viscosity range by four decades. The variation with pressure (P) is smooth and no discontinuity occurs at the point where we began using centrifugal force to accelerate the sphere. The sigmoidal shape of the curve of high-pressure $\log \eta$ against pressure is less pronounced for glycerol than for other glass formers⁹, but $\partial \log \eta / \partial P$ exhibits a clear minimum; this locates the inflection point of $\log \eta$ near 1.5 GPa, in a pressure range consistent with results for other alcohols.

We find that over the entire viscosity range our data for $\eta(P)$ can be accurately fitted using a free-volume equation^{1,2} as shown in Fig. 3. This equation has the form $\eta = A \exp [BV_\infty / (V - V_\infty)]$, where $V - V_\infty$ is the free volume of the liquid (that is, the volume after subtracting that occupied by a molecule and V is the pressure-dependent volume obtained by fitting $P-V$ data¹³ with the Tait equation of state³⁰). This same free-volume equation has been used to model the viscosity of glycerol as a function of temperature. The value of V_∞ for our variable-pressure, isothermal data is $V_\infty = 75.3 \pm 0.4 \text{ \AA}^3$, different from that for the variable-temperature, isobaric data¹⁴ $V_\infty = 112.1 \text{ \AA}^3$. This difference emphasizes the fact that viscosity dependence on free volume is inherently different for isothermal compression than for isobaric contraction, and shows why assignments of physical meaning for V_∞ have been ambiguous^{1,15,16}.

Our value of V_∞ is very close to the molecular volume,

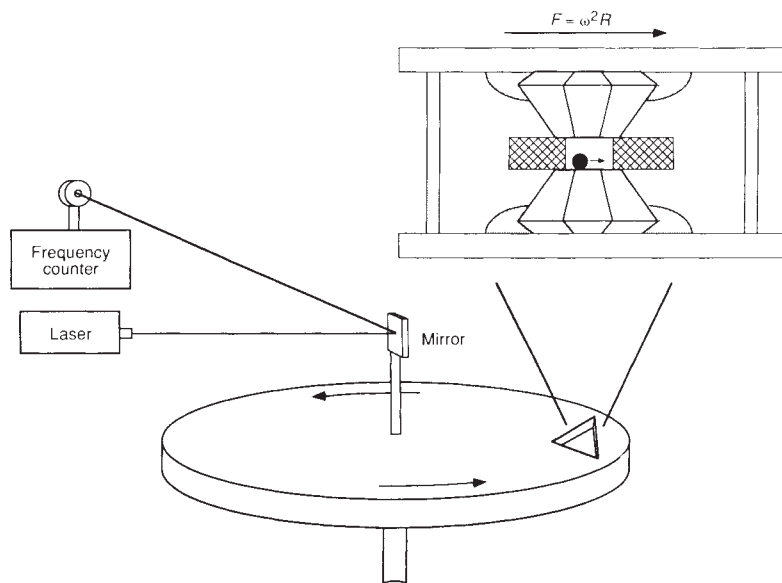


FIG. 1 The centrifugal force viscometer in which a Merrill-Bassett diamond anvil cell containing a small sphere (50 μm diameter) is subjected to a centrifugal force F of up to 1,750 g. The sphere displacement, measured optically before and after application of the force, is proportional to the viscosity through a modified Stokes equation¹⁰ where the total force on the sphere is obtained by integration of the rotation frequency as a function of time. The rolling ball viscometer, using the tangential component of gravity ($<1 g$) to accelerate the ball, is used to measure viscosities between 10^{-1} to 10^6 cP with an accuracy of 4%. The limit at high viscosity is the speed with which the ball moves (10 μm per day at $\eta = 10^6$ cP). With the increased acceleration possible with centrifugal force, we have extended the range of simple rolling ball viscosity measurements by more than three orders of magnitude, while maintaining a precision of $\sim 8\%$. Near $\eta \approx 10^5$ cP, measurements can be made with and without use of the centrifuge; the results are consistent to 5%.

$V_{\text{EHS}} = 73.6 \text{ \AA}^3$, of an effective hard sphere whose diameter is determined from a modified Carnahan-Starling-van der Waals equation-of-state model¹⁷. This assignment is intuitively appealing and widely applicable, with a similar relation found for more than 25 chemically dissimilar compounds (R.L.C., C.A.H. and H.E.K. Jr, manuscript in preparation). Theoretical work¹⁶ on hard-sphere liquids found a similar relationship, but the V_{∞} value from calculated transport properties was considerable larger, 1.57 times the hard-sphere volume, V_{HS} . The difference between V_{∞} and V_{HS} was attributed to the equivalence of V_{∞} and the volume of amorphous closest packing, which includes a packing fraction. This analysis suggests that the molecular volume should be considerably smaller than V_{EHS} to be consistent with V_{∞} . Although this discrepancy is puzzling, we note that the theoretical study was limited to rather low densities and high diffusivities (~ 3 orders of magnitude) whereas our data extend over a considerably larger range of fluidity (> 6 orders of magnitude).

The large difference between V_{∞} for isothermal and isobaric conditions has led others to suggest a temperature and pressure dependence for V_{∞} (refs 14, 18). For isothermal data, our results suggest that a pressure dependence is unnecessary and calculations show that V_{EHS} has a negligible density dependence¹⁷. Although there have been several attempts to determine the temperature dependence of V_{∞} , the use of free volume theory for temperature-dependent viscosity of simple fluids has been superseded by the use of other theories, such as the Adam-Gibbs configurational entropy model¹⁹.

Further evidence that the free-volume model provides an accurate representation of the viscosity is obtained by extrapolating to a viscosity of 10^{15} cP (roughly η at the glass transition for this intermediate liquid²⁰) which then estimates the glass transition pressure as $P_g = 5.0 \pm 0.1$ GPa. This is in reasonable agreement with our determination of $P_g = 4.4 \pm 0.5$ GPa by ruby fluorescence measurements of stress gradients³¹ (data not shown). The model also aids in elucidating the controlling parameters for the inflection point in $\log \eta(P)$. Incorporating the Tait equation of state into the free volume model for viscosity, we find that the volume at the inflection point is given by $V_1/V_0 = V_{\infty}/V_0 + 2/(K'_0 + 1)$ where V_0 is the volume at ambient pressure and K'_0 is the pressure derivative of the bulk modulus. The calculated inflection point for glycerol corresponds to a pressure of 1.4 GPa, which compares well with the

value of 1.5 GPa found in Fig. 2 from a polynomial fit to the data. As can be seen, nonlinearity in the P - V curve (that is, K'_0) completely determines the existence of the inflection point within a physically accessible pressure regime ($V_1/V_0 < 1.0$). The free-volume model fails to describe anomalous high-pressure viscosity data (where, for example, pressure causes the viscosity to decline) for which the Adam-Gibbs configurational entropy model¹⁹ may be useful²¹. However, the functional form (although a simplification of the actual transport mechanism¹⁶)

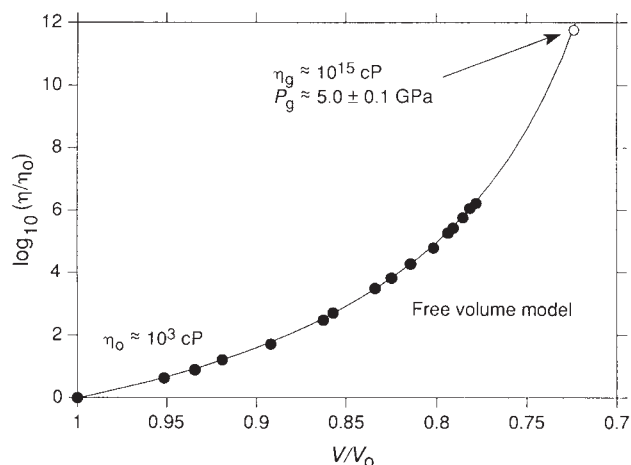


FIG. 3 Reduced viscosity (η_0 is viscosity at ambient pressure) plotted against reduced volume (V_0 is ambient pressure volume). The free volume equation, $\log_{10}(\eta/\eta_0) = BV_{\infty}[1/(V - V_{\infty}) - 1/(V_0 - V_{\infty})]$, with $B = 2.78 \pm 0.08$ and $V_{\infty} = 75.3 \pm 0.4 \text{ \AA}^3$, fits the data over the entire compression range. Extrapolation gives an estimate for the glass transition pressure (5.0 ± 0.1 GPa) in reasonable agreement with that determined from stress gradient measurements (4.4 ± 0.5 GPa).

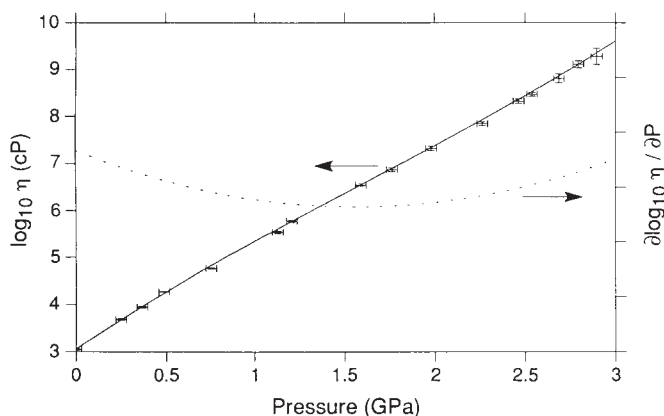


FIG. 2 Pressure dependence of the viscosity of glycerol measured with the rolling ball and centrifugal force techniques and the pressure derivative of the viscosity. Each data point represents the mean of multiple measurements and the calculated standard deviations are shown. These data sets overlap between pressures of 1–1.5 GPa, and agree to within 5%. The pressure derivative is calculated from the smoothed viscosity data; the minimum near 1.5 GPa is in good agreement with the analytical theory described in the text.

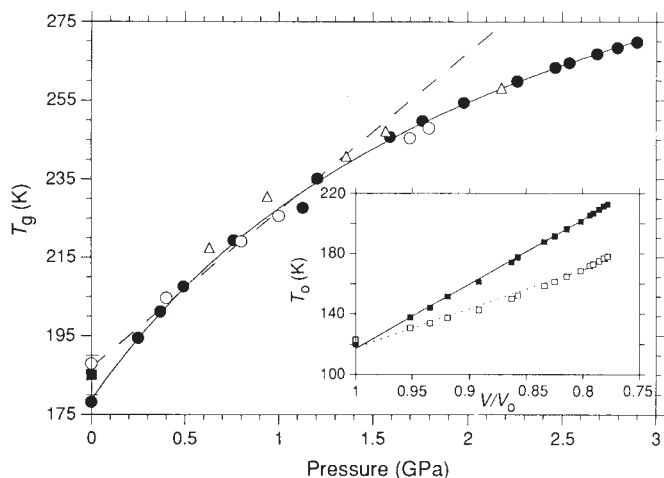


FIG. 4 Glass transition temperature (defined at $\eta = 10^{15}$ cP) as a function of pressure using viscosity data from the present work and the VTF equation $\log \eta = A + B/(T - T_0)$ where $A = -2.48$ and $B = 974$ (ref. 22). The predicted points (●) come from the assumption that A and B are constant^{24,25}, and that $T_g = T_0 + 55$ K as found at ambient pressure²² (the solid line is a guide to the eye). An alternative interpretation is that A and B/T_0 are constant²⁸; then $T_g - T_0$ is a function of pressure. In this case the glass fragility would be pressure independent. Also shown are values of $T_g(P)$ from heat capacity⁸ (○) and dielectric⁶ (△) data. (The heat capacity data were shifted by 20 K to coincide with the accepted value of T_g at ambient pressure.) The non-linearity of dT_g/dP is in contrast to previous reports³⁰ which assumed a linear dependence (dashed line) of $dT_g/dP = 40 \text{ K GPa}^{-1}$. The inset shows that T_0 is a nearly linear function of volume when A and B (■) or A and B/T_0 (□) are constant with V/V_0 evaluated at 295 K.

is fairly successful for a variety of liquids over extended pressure, compression and viscosity ranges, and yields physically reasonable predictions.

The empirical Vogel-Tammann-Fulcher (VTF) equation, $\log \eta = A + B/(T - T_0)$, is an excellent representation for the temperature-dependent viscosity data of glycerol over 12 orders of magnitude²², where T_0 is roughly the Kauzmann temperature²³ (at which the liquid and crystal are isentropic). A modified form of the VTF equation, in which T_0 is assumed to depend linearly on P , has been used to fit high-pressure conductivity^{24,25} and Brillouin scattering data²⁶ but does not describe our viscosity data. Following the assumption that the parameters A and B are constants^{24,25}, independent of both pressure and temperature, then the isothermal high-pressure viscosity data can be used with the VTF equation to calculate $T_0(P)$. Applying the ambient-pressure result²² that $T_g = T_0 + 55$ K, we obtain $T_g(P)$ as shown in Fig. 4. This plot has several important features. First, the resulting $T_g(P)$ are nonlinear with pressure and agree with estimates from high-pressure measurements of heat capacity and dielectric constant^{6,8}. Such nonlinearity is not widely recognized but was theoretically predicted³² and has been seen for other liquids²⁷. We do, however, find a linear relationship between T_0 and volume (inset to Fig. 4).

Alternatively, one can assume that A and the ratio B/T_0 are constant, as found for methanol over a limited pressure range²⁸. This leaves unaltered the nonlinear behaviour of $T_0(P)$, but now $T_g - T_0$ must increase with pressure to agree with experimental data. The nearly linear relationship between T_0 and volume is preserved. The key difference between these two models is whether the fragility parameter B/T_0 (ref. 20) is independent of pressure. This parameter is related to the rate of structural breakdown in the liquid above T_g and is used for the classification of glass-forming liquids. But past studies have not addressed its pressure dependence. Evaluation of this requires

variable-temperature, high-pressure data, and the single available viscosity point for glycerol⁹ suggests that B/T_0 is independent of pressure. This suggests that the combined effects of temperature and pressure on glycerol are such that an isothermal crossing of T_g , at any pressure, would yield a constant configurational entropy change. Further data are clearly required to clarify this issue. □

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Recent change of Arctic tundra ecosystems from a net carbon dioxide sink to a source

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ARCTIC tundra has been a net sink for carbon dioxide during historic and recent geological times^{1–4}, and large amounts of carbon are stored in the soils of northern ecosystems. Many regions of the Arctic are warmer now than they have been in the past^{5–10}, and this warming may cause the soil to change from a carbon dioxide sink to a source by lowering the water table^{11,12}, thereby accelerating the rate of soil decomposition (CO₂ source)^{3,13–15} so that this dominates over photosynthesis (CO₂ sink). Here we present data indicating that the tundra on the North Slope of Alaska has indeed become a source of carbon dioxide to the atmosphere. This change coincides with recent warming in the Arctic, whether this is due to increases in greenhouse gas concentrations in the atmosphere or to some other cause. Our results suggest that tundra ecosystems may exert a positive feedback on atmospheric carbon dioxide and greenhouse warming.

The current, recent and future carbon flux of terrestrial ecosystems is controversial^{16,17}, and at present, the carbon budget does not balance, implying uncertainty as to the current terrestrial carbon flux. It may be that over the past 30–40 years, the terrestrial biosphere has been a net sink for carbon^{11,18} large enough to account for the 'missing carbon' injected into the atmosphere and not accounted for in oceanic uptake or atmospheric storage^{19,20}. Analyses by Tans *et al.*¹⁷ concluded the likelihood of a net terrestrial sink with high-latitude CO₂ sources to the atmosphere.

Northern ecosystems contain up to 455 Gt (petagrams) of C in the soil active layer and the upper levels of the permafrost, or up to ~60% of the ~750 Gt C currently in the atmosphere as CO₂ (refs 1–4). Arctic tundra ecosystems alone contain more than 50 Gt C below ground as dead organic matter. In the historic and recent geological past, rates of carbon accumulation in tundra worldwide have been ~0.1–0.3 Gt C per year^{2–4,12,21,22}. Tussock tundra is estimated to have accumulated carbon at the rate of 23 g C m⁻² yr⁻¹ (0.02 Gt C per year worldwide), wet tundra at the rate of 27 g C m⁻² yr⁻¹ (0.03 Gt C per year worldwide^{2,4}) to 40–120 g m⁻² yr⁻¹ (refs 24, 25).

High-latitude ecosystems are expected to undergo the greatest increases in surface temperature with a doubling of atmospheric CO₂. Surface temperature increases of 4 °C in summer and as much as 17 °C in winter have been predicted for high northern latitudes^{5,23}, providing the potential for large absolute and relative increases in temperature in arctic regions. The importance of permafrost, ice and snow in controlling arctic ecosystem processes also makes the Arctic particularly sensitive to warming²⁴.

Regional warming of surface air temperatures^{5–8} and permafrost temperatures^{9,10} has been experienced over arctic Alaska and Canada and/or central Siberia over the past century. In Alaska, despite complications from heat island effects at

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Barrow²⁶, regional summer warming over the past 70 and 20 years at Barrow and Prudhoe Bay, respectively, seems to be reflected in their temperature records (Fig. 1) and agrees with the general trend in global temperature deviations²⁷⁻²⁹. But although the direction of the change observed on the north slope of Alaska is that predicted by general circulation models and climate reconstructions, the temperature rise cannot definitely be ascribed to greenhouse warming³⁰.

Here we report measurements of whole-ecosystem CO₂ flux measured over five seasons (1983-1985, 1987, 1990) at Toolik Lake and along a latitudinal transect in 1990, from the Arctic Ocean at Prudhoe Bay to Toolik Lake, Alaska (Fig. 2). The initial measurements (1983-1985, 1987) were made at Toolik Lake using a closed, CO₂ and long-term temperature-controlled, null-balance greenhouse chamber (CO₂LT chamber) system³¹. During the summer of 1990, we made diurnal and seasonal measurements of ecosystem CO₂ flux along a north-south transect from the Arctic Ocean at Prudhoe Bay to Toolik Lake, 200 km to the south, using a portable ecosystem cuvette and gas exchange system³².

The repeated summer measurements of diurnal CO₂ flux at Toolik Lake made between 1983 and 1987 indicated a loss of CO₂ from the tussock tundra of 0.72-3.9 g C m⁻² d⁻¹ (Table 1). If we assume a nominal active season of 125 days, these annual rates of CO₂ efflux varied from 53 to 286 g C m⁻² yr⁻¹, reflecting the year-to-year variability in environmental conditions (Table 1). We may have slightly underestimated CO₂ loss by

missing some periods of soil and plant respiration in early spring and late autumn (S. A. Zimov, personal communication).

Diurnal and seasonal patterns of carbon flux were dominated by periods of carbon loss to the atmosphere. For example, Happy Valley shows a seasonal increase in the rate of loss of carbon at 'night' from early season to peak season. This increase is presumably due to increasing soil temperatures, soil aeration and depth of thaw over this period. Peak net ecosystem carbon uptake also increases from early to mid-season, but on no days measured is the carbon balance positive, and soil decomposition and plant respiration always exceed photosynthetic uptake for the diurnal period. By mid-season, despite increases in midday rates of carbon uptake, carbon loss has increased more than has photosynthetic uptake, and the net loss of carbon has increased compared to early season rates. The measurement period was extended in 1991 to cover almost the entire snow-free period, reducing the extrapolation necessary (W.C.O., unpublished data). The results support those presented here and confirm the carbon loss from spring snow melt to autumn freeze-up along the transect measured.

Net seasonal carbon loss to the atmosphere was found at all sites measured. Carbon loss to the atmosphere during the 1990 season generally increased from north to south (Fig. 2), and on the drier areas within a site (Fig. 2, Table 1). The site closest to the coast, Prudhoe Bay Wet, was the wettest site measured (Table 1). The southern tussock tundra sites, Toolik Lake and Happy Valley, were better drained, and had greater thaw depths

FIG. 1 Mean annual summer (June through August) temperatures from 1921 to 1990 at Barrow Alaska and from 1970 to 1990 at Prudhoe Bay Alaska. Represented are 3-yr sliding mean values from the U.S. Weather Service for Barrow, Alaska and from ARCO for the ARCO airport tower at Prudhoe Bay, Alaska.

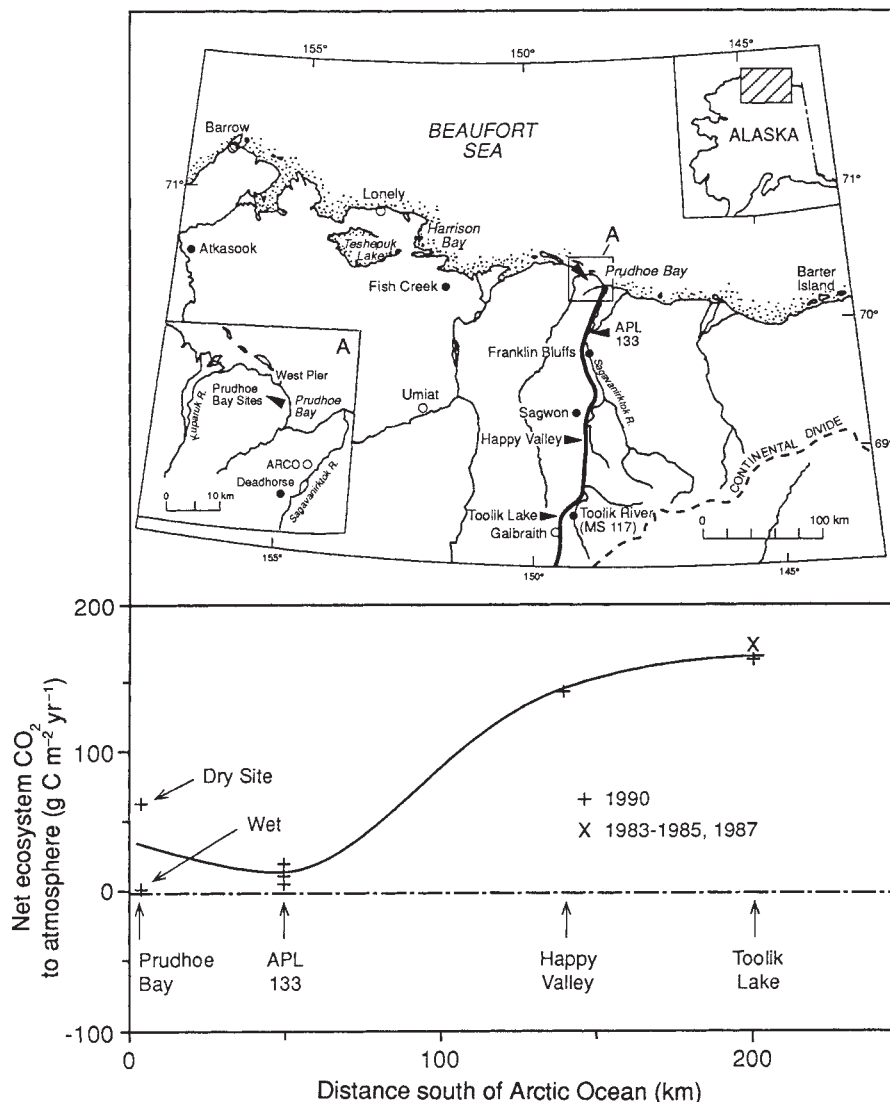


TABLE 1 Characteristics of research sites and measured CO₂ fluxes

Site	Latitude	Longitude	Distance (km)	Elevation (m)	Tundra type	Moisture	Year	Average summer temperature* (°C)	Summer precipitation* (mm)	Average CO ₂ flux (g C m ⁻² d ⁻¹)
Prudhoe Bay wet	70° 22'	148° 45'	2.5	3	Wet sedge	Wet	1990	6.5¶	49§	+0.034
Prudhoe Bay moist	70° 22'	148° 45'	2.5	3	Wet sedge	Moist	1990	6.5¶	49§	+0.66
APL-133 control	69° 50'	148° 45'	50	80	Wet/moist sedge	Moist	1990	10.0‡	69‡	+0.216
APL-133 flooded	69° 50'	148° 45'	50	80	Wet/moist sedge	Wet	1990	10.0‡	69‡	+0.091
APL-133 drained	69° 50'	148° 45'	50	80	Wet/moist sedge	Moist	1990	10.0‡	69‡	+0.39
Happy Valley	69° 08'	148° 50'	140	366	Tussock	Moist	1990	13.1†	50†	+1.155
Toolik Lake	68° 38'	149° 35'	200	732	Tussock	Moist	1990	9.3‡	160	+1.34
Toolik Lake							1983	8.9#	N.D.**	+3.8
Toolik Lake							1984	N.D.**	264	+3.9
Toolik Lake							1985	7.5‡: 6.5#	213	+1.1
Toolik Lake							1987	9.2§	246	+0.72

Positive values of flux indicate CO₂ loss to the atmosphere. Daily CO₂ flux measurements are calculated from diurnal measurements, and the integrated seasonal uptake is averaged to provide an average daily flux for the period of biological activity.

* Summer months from June–August.

† This study, missing 22 days of data.

‡ Toolik River, MS-117 (D. Kane, personal communication).

§ This study.

|| Toolik River, MS-117 (R. J. McClure, personal communication).

¶ National Climatic Data Center, Asheville, NC computed from the mean minimum and mean maximum recorded temperatures.

Toolik River, MS-117 (R. H. Haugen, personal communication).

** N.D. Not Determined.

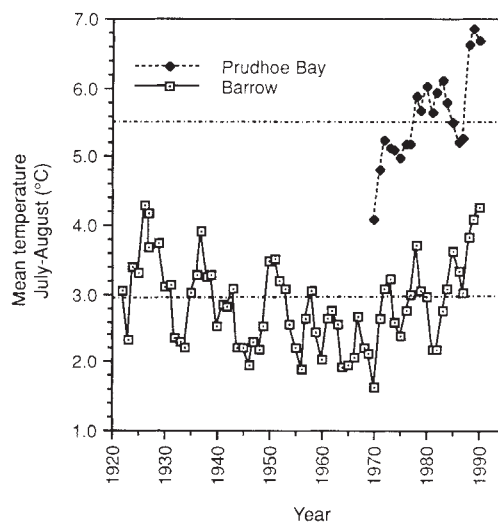
and lower water tables. The average rate of annual CO₂ loss for the tussock tundra sites in 1990 was 156 g C m⁻² yr⁻¹, whereas carbon loss for the wet coastal tundra sites (Prudhoe Bay and APL 133-3) was ~34 g C m⁻² yr⁻¹ (Table 1). The results for the tussock tundra ecosystems during the 1990 sampling season agree well with the initial measurements made between 1983 and 1987 (Table 1) and with subsequent measurements made in 1991 and 1992 (W.C.O., unpublished data). These data indicate a change in carbon balance of the tundra with respect to the atmosphere, in response to the trend in climate warming for the north slope of Alaska.

We feel that the change from carbon accumulation to carbon loss in these ecosystems is not caused directly by the increase in temperature but indirectly, by enhanced drainage and soil aeration, and a decrease in the water table^{11,12}. Decomposition of the soil organic layer in northern peatlands is controlled much more by drainage, and consequently soil aeration, than by temperature^{3,13-15}. Warmer periods in the past that resulted in peat accumulation in the Canadian sub-arctic and Alaskan arctic³³, where there is no evidence of current carbon accumulation³, are thought to reflect the combination of warmer and wetter conditions.

FIG. 2 Site locations on the north slope of Alaska. From north to south: Prudhoe Bay, APL-133, Happy Valley, Toolik Lake (upper panel). Seasonal carbon dioxide flux (as carbon) along a latitudinal gradient from Prudhoe Bay to Toolik Lake, Alaska in 1990 (+), and for four additional years of measurement at Toolik Lake (1983–1985, 1987; ×). The line represents an interpolation of values for 1990. Mean flux for 1983–85 and 1987 is 175 ± 62 g C m⁻² yr⁻¹ (mean ± 1 s.e.). Site differences in soil moisture are noted at Prudhoe Bay and APL 133. The three values at APL 133 represent the three areas of differing moisture availability (drained, control and impounded) studied which resulted in higher, medium and lower CO₂ efflux from the tundra to the atmosphere respectively. For the initial measurements (1983–85, 1987), there were three replicate control chambers running under ambient conditions. Measurements were made every 3 minutes, and averaged and recorded every 6 minutes throughout the day and the season. For 1990, measurements were made from shortly after snow melt to shortly before soil freeze-up in the autumn at each site on each of six replicate plots roughly once every 1.5 h throughout the 24-h measurement period. Seasonal flux was extrapolated to zero at the time of snow melt in the spring and of soil freeze-up in the autumn. The net seasonal flux for each site was determined as the integral under the curve of the seasonal pattern of daily fluxes. There was little variability in daily CO₂ flux rates among the replicates at each site on any day. Because these measurements were made only during the snow-free summer period, they underestimate the seasonal carbon loss to the atmosphere by the presumably small amount of soil and plant respiration that occurs under snow during the unmeasured winter period.

Given the general pattern of warming for the north slope of Alaska and the Canadian arctic^{6-10,34}, the patterns observed along this transect in Alaska may be widespread and the carbon loss huge. If the average rate of loss in tussock tundra of 156 g C m⁻² yr⁻¹ is typical for circumpolar tussock tundra, the loss of carbon to the atmosphere in 1990 from the 0.90 × 10⁶ km² of tussock tundra worldwide² would be 0.14 Gt C per year. Similarly, if the 34 g C m⁻² yr⁻¹ of CO₂ evolution from wet tundra is applied to the 1.00 × 10⁶ km² of wet coastal tundra, another 0.03 Gt C is lost annually to the atmosphere. Adding 0.02 Gt C for efflux from arctic tundra lakes and rivers³⁵ results in a total estimated annual loss of 0.19 Gt C from these three circumpolar arctic surface types. This combined efflux can be compared to the 0.2–0.7 Gt C calculated to be lost to the atmosphere from high-latitude ecosystems¹⁷. Given the large below-ground stores of carbon in arctic, boreal forest and high-latitude bogs, these high-latitude ecosystems could provide a considerable positive feedback on increasing atmospheric CO₂ and global warming.

There are many uncertainties in predicting the long-term effect of global change on arctic ecosystems. The initial response to warming and drying may be a loss of carbon from the system,



whereas at longer intervals, invasion by shrub and tree species may result in increased above-ground carbon storage. Other compensatory processes include cooling of the soil surface (following increased insulation from drying of the soil surface and litter or biomass accumulation from elevated CO₂ and/or other climatic effects). Currently there is no evidence that these compensatory processes are acting or will act quickly. Field and laboratory experiments indicate little or no stimulation of photosynthesis in the Arctic by elevated atmospheric CO₂ (ref. 36). It is likely that global warming will lead to significant outgassing of carbon from wet and moist tundra before other processes reincorporate this carbon into arctic areas. Global warming could accelerate the rate of carbon loss in arctic tundra ecosystems by increasing the depth of thaw, increasing soil drainage and aeration, and increasing rates of soil decomposition and plant respiration more than gross primary productivity.

The recent climate patterns may be part of the normal climate variation or an early indication of greenhouse warming. In either case, it is clear that they have affected the current carbon flux from the arctic ecosystem, and that arctic and boreal forest ecosystems could provide a strong positive feedback on atmospheric carbon dioxide concentration. □

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The transient response of terrestrial carbon storage to a perturbed climate

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MODEL simulations suggest that at equilibrium, global warming driven by higher atmospheric concentrations of greenhouse gases will lead to increased terrestrial carbon storage^{1,2}, implying a negative feedback between the global vegetation/soil system and the atmospheric CO₂ concentration. But changes in vegetation and soil type that result in a net release of CO₂ to the atmosphere (such as those caused by wildfires) could be more rapid than changes that result in a net increase in terrestrial carbon storage (such as species immigration and soil formation), so that in its transient response to climate change, the terrestrial vegetation/soil system could be a net source of carbon to the atmosphere. Here we use two general circulation models^{3,4} to estimate the transient response of the terrestrial surface to a step doubling of atmospheric CO₂. We find that vegetation and soil changes could prove to be a significant source of CO₂ in the first 50–100 years following a climate warming, increasing the atmospheric CO₂ concentration by up to a third of the present level.

Several estimates have been made for the carbon reserves of the terrestrial surface^{5,6}. These estimates are obtained by classifying the vegetation and soils, and then multiplying area estimates for each classification category by the carbon concentration of soil and vegetation in research sites typical of that category. Vegetation and soil classifications have also been

related to climate variables and used in assessments of possible global responses to climate changes^{1,2,7}, for example to estimate changes in potential terrestrial carbon storage. Such calculations presumably estimate a long-term equilibrium response to a climate change. In the case of a 'greenhouse gas' climate warming, the long-term response of the terrestrial surface is to store more carbon^{1,2}.

Here we extend the methodology used by Smith *et al.*² to estimate the transient terrestrial surface response to a step change in climate. In brief, the method of determining present and future carbon reserves was this. (1) Expected current global distributions of vegetation were mapped at a 0.5° × 0.5° (latitude and longitude) resolution using the Holdridge life-zone classification⁸ and a climate database of monthly precipitation and temperature⁹. (2) Estimates of carbon storage per unit area for each Holdridge life zone² were derived for above-ground biomass using data from Olson *et al.*⁵ and for soil carbon from Post *et al.*¹⁰. These estimates were applied to the map produced in step 1 to obtain global terrestrial-surface carbon pools². (3) Simulations of current (1 × CO₂) and 2 × CO₂ climates from the general circulation models developed by the Goddard Institute for Space Studies³ and the Geophysical Fluid Dynamics Laboratory⁴ were used to construct climate change schemes². (4) The climate database was altered according to the changes implied by the climate-change schemes, and steps 1 and 2 (above) were repeated to obtain estimates of carbon storage (presumably after a long period of equilibration) for the altered climate conditions.

To obtain an estimate of the change in source-sink relations between the terrestrial surface and the atmosphere, we developed a simple compartment model of terrestrial carbon reserves (above-ground biomass and soils) and applied this model to the globally mapped vegetation changes in response to the two climate-change schemes. The compartment model used in this analysis parallels the approach used in current

TABLE 1 Carbon pools for GFDL and GISS

Above-ground biomass						
Scenario	Loss		Gain		Net	
	GFDL	GISS	GFDL	GISS	GFDL	GISS
Successional replacement (Rate 0.004 yr ⁻¹)	323.0	292.9	338.7	346.2	+15.7	+53.5
Immigration (Rate 0.001 yr ⁻¹)			122.4	171.8	+122.4	+158.5
Dieback/disturbance (Rate 0.02 yr ⁻¹)	124.1	119.4			-124.1	-119.4
Soils						
Scenario	Loss		Gain		Net	
	GFDL	GISS	GFDL	GISS	GFDL	GISS
Increased decomposition (Rate 0.02 yr ⁻¹)	196.8	213.3			-196.8	-213.3
Successional replacement (Rate 0.004 yr ⁻¹)	87.3	111.3			+87.3	+111.3
Immigration (Rate 0.001 yr ⁻¹)			101.9	102.0	+101.9	+102.0
Total					GFDL +37.9	GISS +146.9

Carbon pools for soils and above ground biomass associated with the changes in vegetation and corresponding soils under the two GCM climate change scenarios. Data represent changes from current pools to those predicted for the $2\times\text{CO}_2$ equilibrium vegetation distribution. Values for above-ground biomass represent either (1) carbon gain from successional replacement of vegetation, (2) carbon loss from vegetation that is being replaced, (3) carbon lost from vegetation affected by dieback and disturbance as a function of increased aridity, or (4) carbon gain from successional replacement which is rate limited by species immigration. Soil carbon values represent the soil carbon gains and losses associated with the changes in vegetation. Losses of soil carbon would result from increased decomposition rates associated with the new climate and vegetation. Gains are dependent on the buildup of organic material from either succession replacement of vegetation, or development of vegetation that is dependent on species immigration. The main vegetation transitions associated with these fluxes are listed in Table 2. All values are in gigatonnes. Although both models predict a net increase in terrestrial carbon storage under equilibrium conditions of increased atmospheric CO_2 the differences in response time of the individual pools mean that the terrestrial biosphere acts as a transient carbon source.

global carbon models¹¹⁻¹⁴. The transitions between Holdridge life zones under current and changed climate were classified by the processes controlling the changes in vegetation and associated soil carbon. For vegetation those processes are: (1) dieback of existing vegetation as a result of increased aridity (accompanied by wildfire); (2) successional replacement of existing vegetation by vegetation predicted for that location under the new climate (natural mortality of the original vegetation fol-

lowed by the establishment and growth of replacing vegetation); and (3) successional replacement limited by the need for immigration of new species. Immigration was deemed necessary if the transition implied a large shift in physiognomic structure or life-form dominance (for example, from tundra or grassland to forest). This definition of immigration-dependent vegetation change is conservative, as most vegetation transitions would involve a considerable taxonomic shift. In those areas where dieback of existing vegetation occurs (process 1), replacement of vegetation predicted to occupy the area under the new climate was calculated on the basis of the successional rate (process 2 or 3). In the case where immigration is required (process 3), we have not included the possibility of a transient vegetation population occupying the sites between the death of existing vegetation and the growth of its replacement.

For process (1) we took the carbon loss per year as a fraction 0.02 of the carbon associated with the original vegetation. For process (2) we took 0.004 yr^{-1} of the carbon associated with the original vegetation as a source, and, in symmetry, 0.004 yr^{-1} of that associated with the new (replacement) vegetation as a sink. For process (3), the rate of development of the replacement vegetation was estimated as 0.001 yr^{-1} . These rates were intended to be conservative, reducing the likelihood of estimating a strong source from the terrestrial surface in the initial stages of the transient carbon dynamics in response to climate change.

For below-ground sources of carbon, the rate at which soil carbon was lost from soils whose organic pool was expected to be lower under the new climate was 0.02 yr^{-1} . Although there is likely to be a lag in soil development as succession proceeds, the rate at which soils accumulate carbon was set at the successional rate (0.004 yr^{-1}). Where the development of replacement vegetation providing organic matter was limited by immigration, the carbon accumulation rate for soils was 0.001 yr^{-1} . As for above-ground rates, these rates were felt to be conservative.

Transfer rates ($1/\text{turnover time}$) were estimated from published values. Transfer rates for above-ground biomass associated with succession were estimated from gap-model simulations¹⁵⁻¹⁹ and relate to the demographic limitations on biomass accumulation. Transfer rates associated with vegetation immigration were estimated from palaeo-studies^{20,21}. Estimates of soil carbon turnover were based on average parameters from global carbon models¹¹⁻¹³. All transfer rates were varied by $\pm 50\%$ to determine the envelope of responses.

When these rates are applied to the changes in vegetation and associated carbon pools as mapped by the Holdridge classification, the terrestrial surface initially behaves as a carbon source in response to both the GFDL and the GISS climate change schemes (Fig. 1). For the GFDL, the source strength is at a maximum of 225 gigatonnes (Gt) carbon at year 134 after the change in climate. The GISS case has a maximum source strength of 150 Gt occurring at year 120. When the transfers of carbon losses and gains are varied independently by $\pm 50\%$, these results are qualitatively unchanged. In all cases, the terrestrial surface behaves as a source in its initial response, although the value and timing of the maximum source strength differ. For the GFDL case, the maximum source strength was between 194 and 240 Gt C with the maximum occurring between 115 yr and 181 yr. The analogous range of responses for the GISS case was 121–163 Gt maxima occurring between 101 and 163 yr. The principal difference in the responses of the two GCMs stems from the differences in the associated patterns of vegetation change².

In general, the results can be directly interpreted on the basis of the transfer rates and the size of carbon pools for the three classes of vegetation dynamics included in the analysis (Table 1). The net increase in carbon storage (both above-ground biomass and soils) for vegetation transitions involving successional dynamics is small for both cases in comparison with the losses from dieback or disturbance and the gains due to

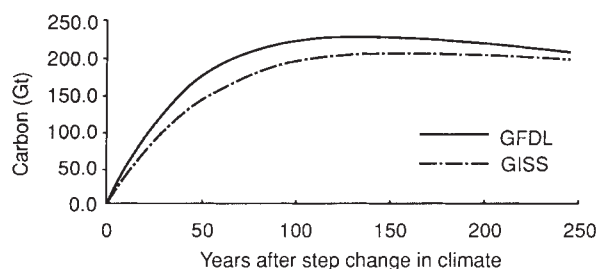


FIG. 1 Net carbon flux to the atmosphere from the terrestrial surface in response to a step climate warming. The fluxes under the two schemes are derived from the changes in vegetation and soils associated with climate change as predicted by two general circulation models for a doubled- CO_2 atmosphere.

TABLE 2 Transitions in Holdridge life zones

GFDL			GISS		
Above-ground biomass			Above-ground biomass		
Immigration			Immigration		
From	To	Net change	From	To	Net change
Polar desert (0.4)	Tundra (9.3)	+8.9	Polar desert (0.7)	Tundra (10.9)	+10.2
Polar desert (0.3)	Boreal forest (18.4)	+18.1	Polar desert (0.1)	Boreal forest (4.8)	+4.7
Tundra (21.4)	Boreal forest (75.5)	+54.1	Tundra (18.5)	Boreal forest (69.7)	+51.2
Subtropical/Tropical Dry Forest (8.9)	Tropical mesic forest (23.7)	+14.8	Subtropical/tropical dry forest (10.5)	Tropical mesic (27.2)	+16.7
Dieback/disturbance followed by succession			Other (40.2)	(45.9)	+5.7
From			From		
To	Net change		To	Net change	
Boreal forest (62.9)	Cool temperate Steppe (20.2)	-42.7	Boreal forest (16.2)	Cool temperate steppe (5.4)	-10.8
Subtropical/tropical mesic forest (27.4)	Subtropical/tropical dry forest (14.9)	-12.5	Cool temperate mesic forest (14.5)	Warm temperate dry forest (8.2)	-6.3
Temperate mesic forest (27.7)	Temperate/subtropical dry forest (16.6)	-11.1	Subtropical dry forest (29.2)	Subtropical/tropical savanna (23.2)	-6.0
Other (6.1)	(5.0)	-1.1	Subtropical mesic forest (33.1)	Tropical dry forest (17.8)	-15.3
Successional replacement			Tropical mesic forest (2.6)	Tropical dry forest (1.0)	-1.6
From			From		
To	Net change		To	Net change	
Subtropical mesic forest (59.9)	Tropical mesic forest (92.8)	+32.9	Other (46.0)	(11.7)	-34.3
Warm temperate dry forest (9.9)	Subtropical dry forest (14.0)	+4.2	Successional replacement		
Subtropical savanna (7.3)	Tropical dry forest (10.9)	+3.6	From		
Boreal forest (67.4)†	Cool temperate forest (48.4)	-19.0	To	Net change	
Other (121.8)	(115.9)	-5.9	Subtropical mesic forest (76.3)	Tropical mesic forest (121.4)	+45.1
Soils			Warm temperate mesic forest (26.6)	Subtropical mesic forest (36.8)	+10.2
Immigration			Boreal forest (64.0)†	Cool temperate forest (47.8)	-16.2
From	To	Net change	Other (56)	(91.7)	+35.7
Polar desert (0)	Tundra (74.5)	+75.2	Soils		
Polar desert (0)	Boreal forest (33.9)	+26.7	Immigration		
Other net changes associated with vegetation transitions			From	To	Net change
From			Polar desert (0)	Tundra (94.9)	+94.9
To	Net change		Polar desert (0)	Boreal forest (7.1)	+7.1
Tundra	Boreal forest	-45.6	Other net changes associated with vegetation transitions		
Tundra	Cool temperate forest	-29.3	From		
Boreal forest	Cool temperate steppe	-14.8	To	Net change	
Boreal forest	Cool temperate forest	-29.3	Tundra	Boreal forest	-66.1
Cool temperate forest	Warm temperate dry forest	-13.6	Boreal forest	Cool temperate forest	-7.1
Cool temperate forest	Warm temperate mesic forest	-7.0	Cool temperate steppe	Warm temperate scrub desert	-7.1
Cool temperate steppe	Warm temperate thorn steppe	-13.4	Cool temperate steppe	Warm temperate dry forest	-6.0
Subtropical dry forest	Subtropical/tropical savanna	-9.4	Cool temperate forest	Warm temperate dry forest	-11.3
Other		+52.9	Cool temperate forest	Warm temperate mesic forest	-9.5
			Warm temperate dry forest	Subtropical dry forest	-13.2
			Subtropical dry forest	Tropical dry forest	+34.0
			Subtropical mesic forest	Tropical mesic forest	+36.0
			Other		+2.3

Chief transitions in Holdridge life zones associated with the net fluxes in above-ground biomass and soil carbon pools shown in Table 1. Transitions have been categorized into (1) successional, (2) dieback/disturbance followed by vegetation succession, (3) successional replacement dependent on the long-distance immigration of species. Relevant pool sizes associated with a life zone are shown in parentheses. Some life-zone designations represent aggregations*. All values are in gigatonnes.

* Mesic forest is the aggregation of moist, wet and rain forest life zones; savanna is the aggregation of thorn steppe and very dry forest; tundra is the aggregation of Moist, wet and rain tundra.

† Transition represents an increase in aridity (for example rain to moist forest).

transitions dependent on vegetation immigration. It is the difference in the response times of these two latter pools that determines the dynamics of the atmospheric-terrestrial biosphere transfer. Overall, the robust result that the terrestrial surface behaves as a carbon source under an abrupt climate warming arises because vegetation and soils changes that result in a net release of CO₂ to the atmosphere (such as conversion of forests to grassland) are potentially rapid in comparison with changes that result in a net increase in terrestrial carbon storage (such as conversion of polar desert to tundra and boreal forest). Carbon pools and net changes in carbon storage for both above-ground biomass and soils for principal vegetation transitions under the two climate-change schemes are shown in Table 2.

The analysis we have presented is simplistic, but shows the importance of considering the timescales of vegetation change when analysing the potential response of terrestrial carbon storage to a changed climate. Although the equilibrium analysis² suggests a net increase in potential carbon storage, there is the potential for a sizeable net transfer of carbon to the atmosphere when the vegetation dynamics necessary to achieve the equi-

ilibrium conditions are examined. Our analyses assume a step change in climate, in that the vegetation transitions were defined from climate-change schemes based on 2×CO₂ equilibrium model runs of the two GCMs. However, the rates of carbon transfer are based on estimates of the time required for those transitions in vegetation and soil properties to occur. In that sense the results represent a transient analysis. The degree to which the results would differ if a transient climate-change scheme were used would depend on the rate of climate change relative to the rates of transfer associated with the vegetation and soil processes (species dieback, species immigration, succession and decomposition). Whichever is the slower process, that process will control the rates of CO₂ flux.

For our analyses we have assumed a set rate of transfer associated with each class of vegetation dynamics. These rates will obviously vary as a function of the vegetation and climate conditions (such as temperature and precipitation) at each location. A detailed analysis using specific rates of response for important terrestrial ecosystems must await a large-scale coordinated ecological research programme. □

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Active volcanism beneath the West Antarctic ice sheet and implications for ice-sheet stability

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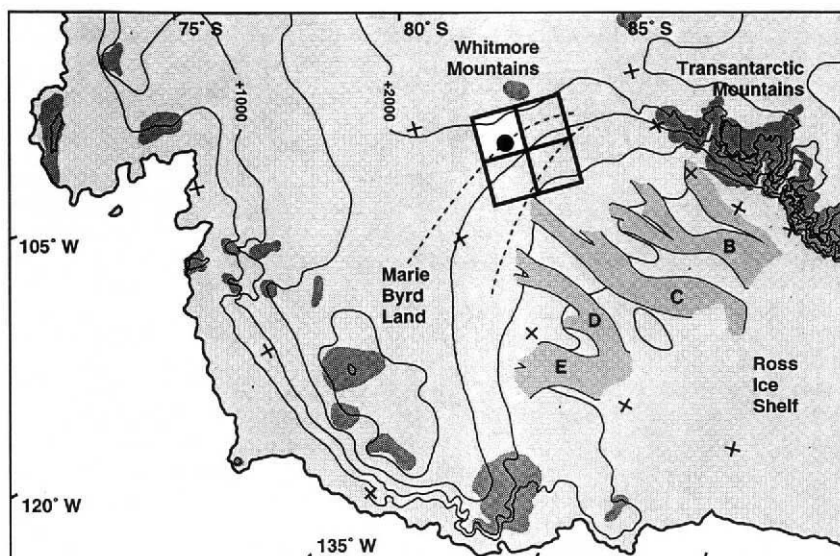
It is widely understood that the collapse of the West Antarctic ice sheet (WAIS) would cause a global sea level rise of 6 m, yet there continues to be considerable debate about the detailed response of this ice sheet to climate change^{1–3}. Because its bed is grounded well below sea level, the stability of the WAIS may depend on geologically controlled conditions at the base which are independent of climate. In particular, heat supplied to the base of the ice sheet could increase basal melting and thereby trigger ice streaming, by providing the water for a lubricating basal layer of till on which ice streams are thought to slide^{4,5}. Ice streams act to protect the reservoir of slowly moving inland ice from exposure to oceanic degradation, thus enhancing ice-sheet stability. Here we present aerogeophysical evidence for active volcanism and

associated elevated heat flow beneath the WAIS near the critical region where ice streaming begins. If this heat flow is indeed controlling ice-stream formation, then penetration of ocean waters inland of the thin hot crust of the active portion of the West Antarctic rift system could lead to the disappearance of ice streams, and possibly trigger a collapse of the inland ice reservoir.

A West Antarctic rift system dominates the region of low-lying topography beneath the WAIS, the Ross Ice Shelf and the Ross Sea⁶ (Fig. 1). This region has experienced several periods of lithospheric extension beginning as much as 175 Myr ago, and possibly continuing through to the present⁷. The evidence for extension in the West Antarctic rift system is best developed in the Ross Sea, where the lithosphere is characterized by thin crust and sedimentary basins as well as recent volcanics^{8,9}. Both Marie Byrd Land and the Transantarctic Mountains are the sites of currently active volcanoes¹⁰. Active volcanism has not been identified within the Ellsworth–Whitmore crustal block, beneath the Ross Ice Shelf or beneath the WAIS. Because of the paucity of outcrop and the difficulty in conducting large-scale ground-based geophysics, few constraints exist on the nature and extent of the extensional processes that may dominate the low-lying topography beneath the WAIS.

As part of a programme entitled Corridor Aerogeophysics of the Southeastern Ross Transect Zone (CASERTZ), we have developed an aerogeophysical platform to study the interaction of geological and glaciological processes in West Antarctica. The aerogeophysical surveying during the 1991–92 field season (Fig. 1) revealed a distinct depression in the surface of the WAIS. This depression is underlain by a peak in the subglacial topography that is associated with a unique magnetic signature (Fig. 2). Located northwest of the Whitmore Mountains

FIG. 1 Map of a portion of the West Antarctic ice sheet¹⁷ with the ice sheet and ice shelves lightly shaded. The South Pole is marked by a box in the upper right corner and the surface elevations are shown with 500-m contours. The approximate regions of exposed bedrock are darkly shaded while the intermediate shading indicates the ice streams of the interior Ross embayment between Marie Byrd Land and the Transantarctic Mountains. The ice streams are identified as B through E from south to north and the western Byrd subglacial basin is located between Marie Byrd Land and the Whitmore Mountains. The West Antarctic rift system is considered to be the region bounded by Marie Byrd Land to the north and by the Transantarctic Mountains to the south and west. In the southeast, it is thought to be bounded by the elevated Ellsworth–Whitmore crustal block which includes the Whitmore Mountains. The CASERTZ aerogeophysical surveying of 1991–92 was accomplished within the four highlighted blocks; the survey block indicated by the white square contains the north–south profile of Fig. 2 and encompasses the area shown in Fig. 4. For each one-degree survey block (111 km by 111 km) aerogeophysical observations were made along orthogonal north–south and east–west profiles with a 5.3-km line spacing for each orientation. Given the average ice thickness of 2–3 km and a survey elevation of 0.5–1.5 km, this line spacing optimizes the recovery of topography from the ice-penetrating radar and unaliased gravity and magnetic measurements. The darkened



circle within the survey area indicates the location of active subglacial volcanism. The dashed lines encompass the region thought to contain the inland edge of hot actively extending lithosphere inferred from the subglacial topography of Drewry¹⁷.

(81° 52.6' S 111° 18.1' W), this feature is near the proposed southern flank of the rift system, and is ~100–200 km east and upslope of the initiation of ice streaming. The subglacial peak, which is 6 km wide at the base, rises 650 m above the surrounding topography to within 1,400 m of the ice surface (Fig. 2b, Fig. 3a). A blowup of the radar image (Fig. 3b) documents a steep-sided (12° slope) and slightly asymmetric morphology. Evaluation of nearby north-south and east-west profiles shows that this peak stands isolated as a central edifice in a 30-km region

of elevated topography that is depressed in the center and bounded by a rim 100–200 m in height. The elevated region and its central edifice are characterized by a 600-nT positive magnetic anomaly, 40 km in diameter (Fig. 2c, Fig. 4a). The gravity signature is a 10-mGal anomaly centred over the edifice (Fig. 2d). The ice surface overlying the central edifice is characterized by a 48-m depression, ~6 km across and offset slightly to the south of the peak (Fig. 2a). The form of this surface depression is constrained both by the east-west profile, which intersects Fig. 2 at 68 km, and by satellite imagery. The orthogonal profile shows that this recess extends 12 km perpendicular to the profile of Fig. 2 and begins 2.5 km to the east, and upslope of the peak. The depression in the ice-sheet surface is visible as a closed feature on the advanced very-high-resolution radiometer (AVHRR) satellite image of the survey area (Fig. 4b).

We interpret the central edifice to be a recently active volcano associated with the hypothesized flank of the West Antarctic rift system along the Whitmore Mountains. The absence of the peak in the radar data from adjacent lines indicates that the proposed volcano is a cone-shaped feature and not a linear ridge. We further interpret the rim ringing the broadly elevated region as the signature of a 23-km-wide caldera. The large-amplitude, long-wavelength magnetic anomaly indicates that the entire elevated region is a volcanic construct.

The depression in the ice surface just south of the central edifice provides evidence for recent volcanic activity. In our survey area the ice flows down a western dipping slope (Fig. 1) largely perpendicular to the profile of Fig. 2. Thus the surface depression is not directly downstream of the central edifice and cannot be a simple response to its presence. The anticipated response of the ice flowing over an elevated topographic feature would be a bump on the ice surface upstream, possibly associated with a depression downstream¹¹. Contrary to this prediction, the depression that we observe is not only offset from the central edifice but extends eastward and upstream. For these reasons, the depression in the ice surface is not a response to sub-ice topography but instead indicates an increased flux of ice into a region of anomalous basal melting.

To estimate the basal heat flow, we conservatively assume that ice is entering the depression only from the north and south along about one-half of its length (6 km), and that the inward slope at the edges of the depression is ~0.005. This slope assumption ignores the local increases caused by the bumps bounding the depression. A simple mass-flux calculation¹² based on the flow law for ice at -20 °C shows that, to maintain this slope, ~0.07 km³ of ice must be removed each year from the base of an ice sheet 2,000 m in thickness. The power required to melt this ice is 700 MW. If the central edifice represents an isolated hydrothermal vent 6 km in diameter, the heat flow at this vent is 25 W m⁻², whereas if the source area is the size of the surface depression itself (~6 × 12 km), the heat flow is 10 W m⁻². This range of 10–25 W m⁻² exceeds typical continental heat flow by three orders of magnitude but is consistent with observations from other ice-covered volcanoes. In Iceland, the heat flux from a single caldera, estimated from the volume of water produced by eruptive events¹³, is 50 W m⁻². In Alaska, accumulation rates and the internal properties of the ice cap on top of the active caldera of Mount Wrangell¹⁴ were used to estimate a heat flux of 7 W m⁻².

Active subaerial and submarine volcanism has been documented within the West Antarctic rift system¹⁰. In Marie Byrd Land, large shield volcanoes 10–35 km in diameter and 1–2 km in elevation are often characterized by summit calderas of ~6 km in diameter. The narrow central edifice emplaced on the broad constructional feature we have identified is morphologically distinct from these subaerial Marie Byrd Land volcanics. The submarine volcanoes of the Ross Sea are similar in form to the central edifice although they are distinguished by shorter wavelength magnetic anomalies (less than 10 km). We believe the steep slope of the central edifice (12°) indicates that it was

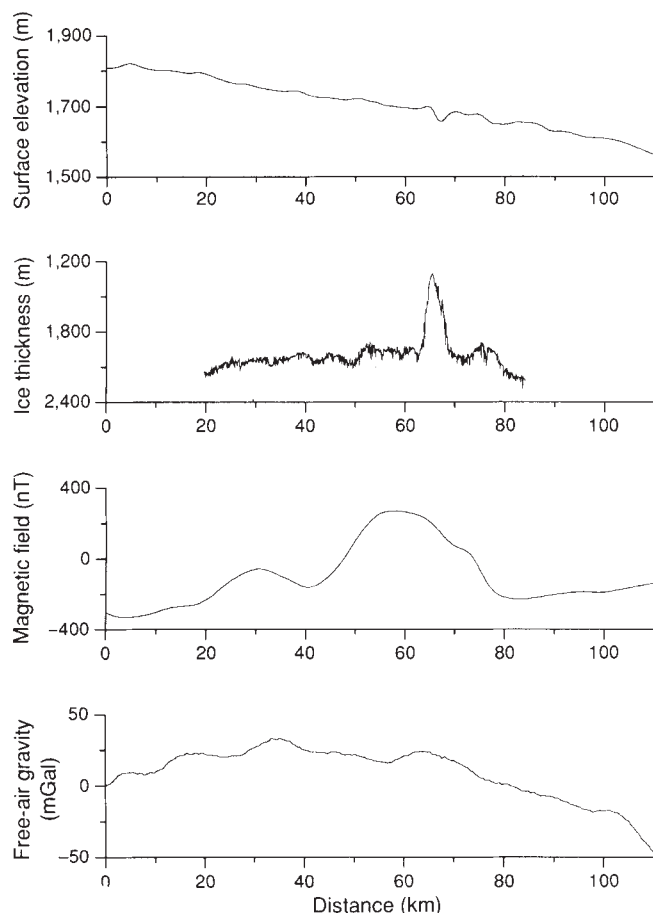


FIG. 2 Aerogeophysical observations made along one of our north-south profiles. The location of this profile is indicated in Fig. 4. These observations were collected from a de Havilland Twin Otter equipped with an ice-penetrating radar, a proton-precession magnetometer, an airborne gravity system and a laser altimeter. The aircraft is navigated by a local radio-transponder network while differential positioning techniques based on the Global Positioning System (GPS) satellites are used for recovering high-resolution horizontal and vertical positions. Attitude information from an inertial navigation system is used to correct the laser altimetry and a digital pressure transducer is used to recover vertical positions and accelerations in the absence of satellite positioning. *a*, Surface elevation from laser-altimeter measurements which have been corrected for aircraft attitude changes and combined with the altitude of the aircraft determined using differential satellite positioning techniques. The laser altimeter profiling of the ice surface is accurate to ~1 m. The anomalous depression in the ice surface is located at 67 km. *b*, Ice thickness automatically picked from the ice-penetrating radar observations of Fig. 3. The 60-MHz ice-penetrating radar can recover sub-ice topography with an accuracy of ~10 m through 2–3 km of comparatively warm West Antarctic ice. The peak of the central edifice is at 66 km and the rim of the proposed caldera intersects the profile at 53 and 76 km. *c*, Total magnetic field, accurate to several nanoTesla, corrected for ionospheric variations using observations from a fixed base station and with the geomagnetic reference field removed; the large anomaly between 40 and 80 km is strongly correlated with the volcanic construct which includes the caldera and central edifice of *b*. *d*, Free-air gravity profile, accurate to better than 5 mGal.

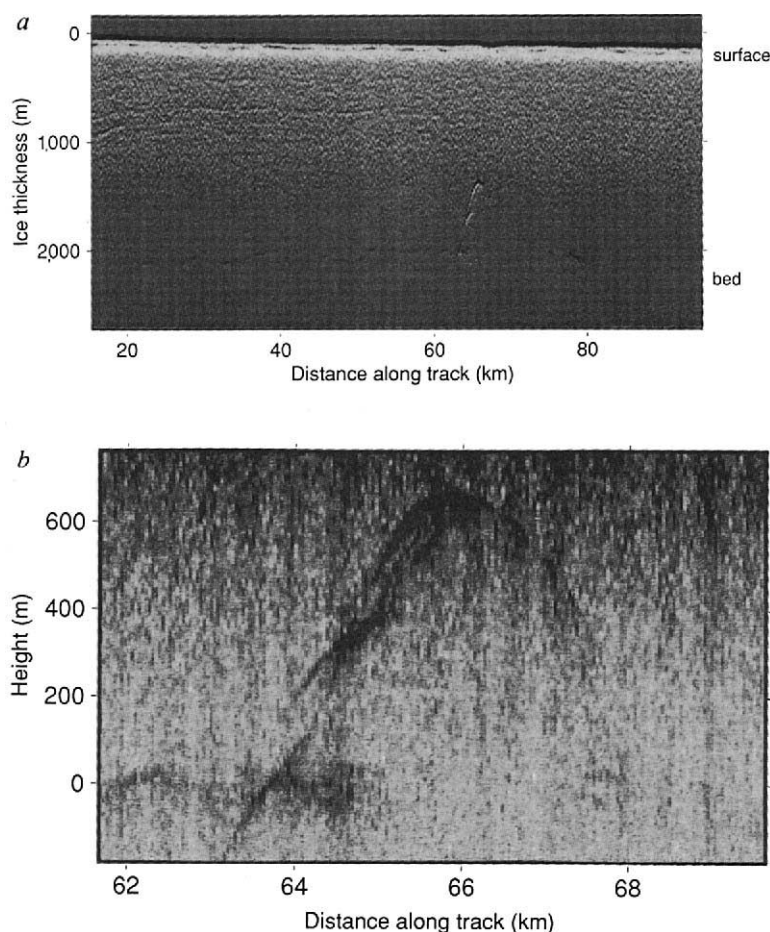


FIG. 3 Ice-penetrating radar observations made along the north-south profile designated by the arrows in Fig. 4. The horizontal scales are referenced to Fig. 2. *a*, The original observations used for Fig. 2*b*. Each of the 1,560 time-differentiated sweeps shown here represents the integrated result of 2,048 radar transmissions. The proposed volcanic construct between 50 and 80 km as well the central edifice and its associated depression in the ice surface are clearly visible. In West Antarctica, radar echoes are often observed for thicknesses greater than 2,500 m so the weak signal strengths for thickness greater than 2,200 m probably result from an anomalously warm ice column. *b*, An expanded non-differentiated view of the central edifice. The north side of this steep-sloped feature is rugged as indicated by the tails of diffraction hyperbolae. The relatively smoother south side is characterized by weaker signal strengths which may indicate increased attenuation of radar transmissions resulting from elevated ice temperatures on this side of the proposed caldera.

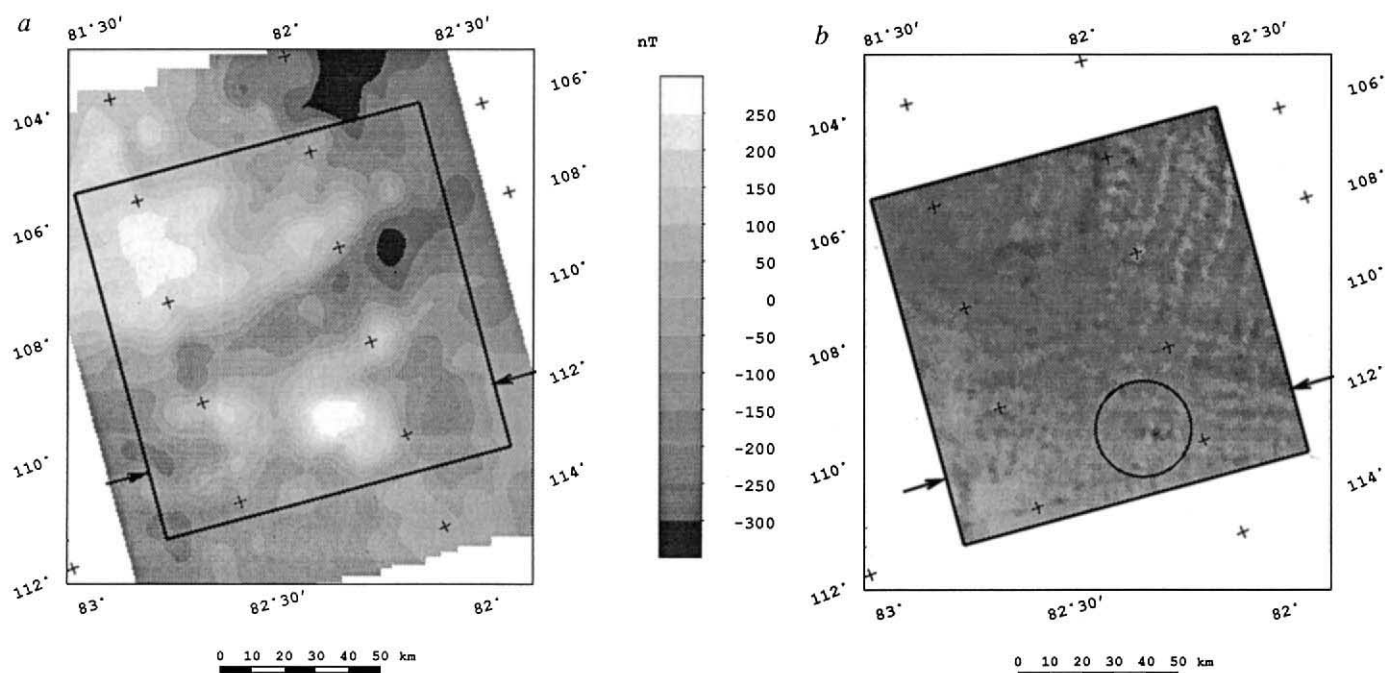


FIG. 4 *a*, Map of the total intensity of the magnetic field for the white survey block of Fig. 1 with a 50-nT contour interval. The arrows show the location of the profile used for Figs 2 and 3. A large kidney-shaped anomaly characterizes the volcanic construct that we have identified. The reduced amplitude for the lobe of the anomaly southwest of the central edifice likely indicates elevated crustal temperatures on this side of the proposed caldera.

b, AVHRR satellite imagery for 13 November 1991 at 0719 GMT over the same region as *a*. This image is illuminated from the south-southeast and AVHRR has a resolution of 1.1 km. A circle roughly the size of the proposed volcanic construct is positioned to include most of the kidney-shaped anomaly of *a*. The depression in the ice surface to the south of the central edifice is indicated by darker shading.

extruded into the ice or possibly underwater before the development of the WAIS.

Other satellite imagery over the ice streams and their catchment basins suggests that the volcano we have identified is not an isolated feature beneath the WAIS. Strikingly circular features in the Landsat images from ice stream E in West Antarctica¹⁵ might also be interpreted as volcanic constructs. The main implication of active volcanism beneath the ice is that elevated geothermal flux provides an important control on the dynamics of the WAIS. Spatial variations in geothermal flux will regulate the water supply available for saturating the sediment responsible for ice streaming. If the rifting process is providing the melt water and sediment required for ice streaming, then the ice streams may be unable to migrate beyond the edge of the active portion of the West Antarctic rift system. This edge would be a major geological boundary between thin hot actively extending lithosphere and cold inactive lithosphere. We believe such a boundary exists between the lithosphere of the interior Ross embayment, the relatively flat low-lying area between Marie Byrd Land and the Transantarctic Mountains, and the region dominated by the Ellsworth–Whitmore crustal block and the Byrd subglacial basin¹⁶. If the downstream terminus of the ice streams were to retreat to this geological boundary, then the reservoir of slow-moving inland ice would be exposed to the ocean without the buffer of the ice-stream system; this would almost certainly represent an unstable situation. Therefore, the character of the lithosphere within the central West Antarctic rift system and, specifically, the distribu-

tion of elevated heat flow and sedimentary basins, represents a fixed boundary condition for the WAIS that is independent of global climate yet could be responsible for triggering the collapse of the ice sheet. □

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Seismological mapping of fine structure near the base of the Earth's mantle

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THE Earth's core–mantle boundary (CMB) juxtaposes liquid iron and crystalline silicates, and is a region of large vertical thermal gradients. The D'' region, which extends up to 200–300 km above the CMB, often has elevated shear-wave velocity and suggestions of lateral variations in structure¹. Recent improvements in our ability to assemble and analyse records from regional seismic networks have allowed us to examine long profiles of travel times, amplitudes and waveforms from more than a thousand short-period seismometers². We observe, across Canada and the United States, P waves that have grazed the CMB from the powerful nuclear test in Lop Nor, China, on 21 May 1992. First-arrival travel times and large secondary arrivals are consistent with a 1.5% compressional velocity increase with depth ~130 km above the CMB—about half the thickness of D'' in this locality³. Our observations, together with evidence for the absence of such a thin, fast layer in neighbouring regions, suggest the presence of lateral heterogeneity in composition or phase at the base of the mantle.

Discrimination between the various thermal and compositional explanations of heterogeneity at the base of the mantle requires knowledge of variations in the velocity of both compressional (P) and shear (S) waves. The D'' layer is laterally heterogeneous^{1,4–8}; some studies find a sudden increase with depth in the P- and S-wave velocities 200–300 km above the CMB^{1,6–11}, whereas others infer smoother velocity variations^{6,12}. A P-wave velocity decrease in the deepest 100 km of the mantle has also been inferred^{10,13,14}, suggesting a thermal boundary layer^{15,16}.

The nuclear explosion at Lop Nor, China, with a yield of 0.66 Mt (megatonnes high explosive equivalent) (21 May 1992, 41.55° N 88.84° E, 1.6 km deep, $m_b = 6.5$; L. Gao and T. C. Wallace, personal communication), provided a large, impulsive P-wave source. It has been more than 10 years since the last explosions of this size, and the seismic networks at that date were much more sparse. The size of the Lop Nor explosion allows us to see short-period P waves diffracting into the distances of the core shadow, and its brief duration allows us easily to isolate multiple arrivals separated by only 1–2 s. By assembling many records from a single event, we avoid the problem faced by previous researchers of how to combine records of an array of events, with generally unknown origin-time errors, mislocations and amplitude calibration between events.

We use the digital short-period seismograms from 14 regional networks in the United States and Canada¹⁷ to investigate the structure above the CMB. The P waves graze the core between the sections of the CMB beneath northern Alaska and Greenland (Fig. 1). The 477 seismograms with the lowest background and signal-generated noise levels were chosen from a total of 1,062 available.

Figure 2 shows seismic sections of stations in three narrow azimuthal ranges. The P wave is simple in the range from 80° to 92°. Later arrivals appear in the distance range from 92° to 103°, which vary in timing and amplitude with azimuth from the explosion. A clear arrival with slightly smaller amplitude follows the P wave by 1–3 s in each profile. Much less distinct later arrivals are seen for profiles across the eastern third of North America. This secondary arrival is not due to scattering near the receivers, as it is absent in earthquakes that we have examined and it is similar for nearby stations. It does not arise from source complexity, as it is absent in the distance range from 80° to 92°.

The secondary arrival is travelling 0.25 s deg⁻¹ more slowly than the initial P arrival. Figure 3a presents the first and secondary arrival times. The pattern is typical of an abrupt increase in velocity with depth: the slowness (reciprocal apparent velocity) of the first arrival decreases abruptly from 5.0 s deg⁻¹ in the

distance range 80° to 90° to 4.5 s deg^{-1} in the distance range 90° to 103° .

Ray tracing and reflectivity modelling verify that this arrival can be explained as a wide-angle reflection from a thin, fast layer at the base of the mantle. The reflections from the core (PcP) and from the top of the layer should be too small to be observed. The secondary arrival's slowness at the surface in a radial velocity structure would be $r_t/r_0 v_r$, whereas the core-grazing P wave has the slowness $r_b/r_0 v_b$. v_r is the velocity just above the reflector, v_b is the velocity just above the CMB, r_0 is the radius of the Earth, r_t is the radius to the reflector and r_b is the radius of the core. The difference in slowness between the wave grazing the top of the thin layer and the wave grazing the CMB is $\Delta v r_b / v_b^2$ plus $\Delta r / v_b$, where Δv is the velocity contrast across the top of the thin layer and Δr the thickness of the layer, in the limit of small thickness and velocity contrast.

We estimate that two-thirds of the difference in slowness between the two arrivals is caused by the difference between r_t and r_b with the following argument. If the refracting layer is 130 km thick, then the layer is 1.5% faster than the mantle just above. A smaller velocity contrast and thicker layer would not produce the 6–10° range of observation of the secondary arrival. A larger velocity contrast across the refracting interface would require a thinner layer, but the thickness of 130 km is consistent with the time difference between the arrivals and the cross-over of the two phases near 89° .

The strength and coherence of arrivals shown in Fig. 2 suggest that a single interface is the primary structure. Some energy that corresponds to the wide-angle reflection from roughly 250 km above the CMB, perhaps the top of D'', appears 4–10 s behind the diffracted P wave in the distance range from 93° to 98° , but this observation is much less clear. The large amplitude of the

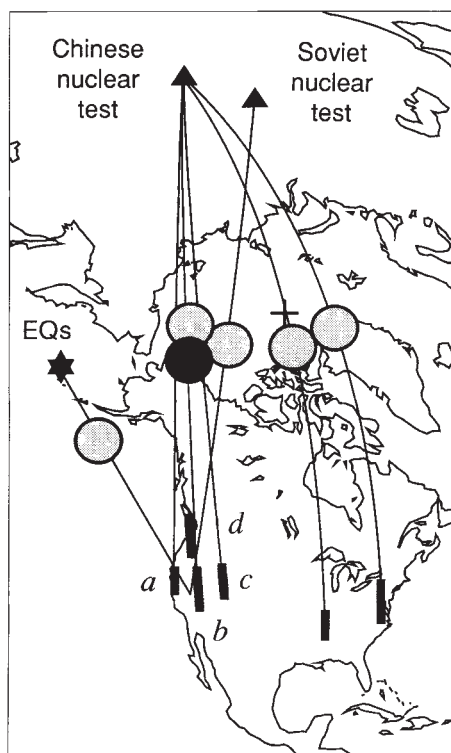


FIG. 1 The great circle paths of the rays sampling the CMB from nuclear tests in China and the Soviet Union and earthquakes in the Andreanof Islands. The heavy lines labelled *a*, *b*, *c* and *d* correspond to the profiles shown in Fig. 2. The dark region shows a 130-km-thick layer at the base of the mantle, the light regions do not show such a structure. The size of the circles, a few hundred kilometres, roughly indicates the areas of the core-mantle boundary sampled. The resolution is difficult to specify, as it depends on unresolved details of the structure.

secondary arrival would be difficult to produce by scattering from low-contrast structures of dimension less than the 300-km path on the CMB sampled by this study. Weak layering is the most likely explanation for the secondary arrival in Figs 2 and 3. The measurement of a significantly larger thickness for D'', 200–300 km, in the region that we sample³ suggests that we are observing an additional layer. The 1,500-km Fresnel zone of the long-period shear waves that image the top of D'' (ref. 3) leaves open the possibility of a local depression in the top of D'' to 130 km above the CMB; however, the paucity of wide-angle P-wave reflections that would indicate normal D'' thickness across our large array, argues against this explanation.

Narrow azimuth profiles

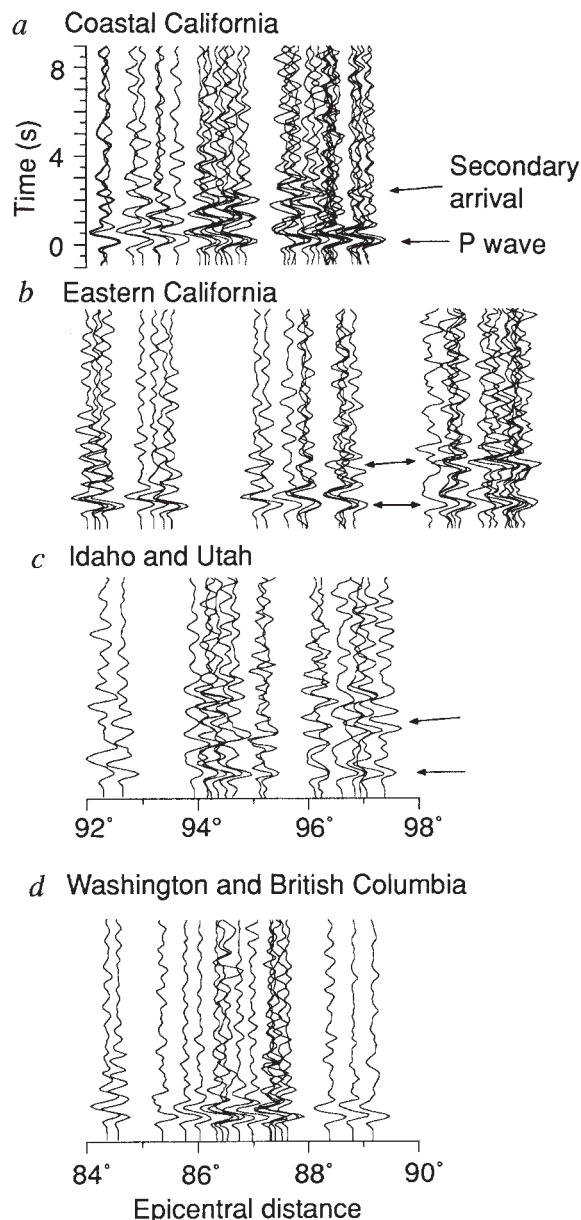


FIG. 2 Seismogram record sections from the Chinese nuclear test. The seismograms have been aligned so that the initial P waves arrive simultaneously and are plotted trace-normalized. The waveforms include the instrument response. First, sections for narrow source-to-receiver azimuthal ranges are shown for the distance range 92° to 100° . *a*, The 36 receivers with azimuths between 24.1° and 24.5° . *b*, The 32 receivers with azimuths between 22° and 23° . *c*, The 17 receivers between 15.7° and 16.5° . *d*, The 18 receivers for the distance range 84° to 90° between the azimuths 20° and 20.5° .

An irregular structure is suggested by the variations in amplitude and timing of the secondary arrival. The arrival varies in amplitude and distance range of observability across North America (Fig. 2) and is absent for some of the stations in Nevada between profiles *b* and *c* that are not shown. The arrival is roughly 0.5 s earlier to the west (azimuths 24° to 25°, Fig. 2a) than to the east (azimuths 15.5° to 17°, Fig. 2c). This difference corresponds to variation of 15–30 km in the reflector depth across a span of 250 km at the CMB. Irregularity or lateral intermittence is also suggested by the lack of secondary near-critical reflections in the distance range 80° to 90°, because for radial layering, such arrivals would accompany the observed wide-angle reflections. It is possible, however, that irregularity in boundary depth or seismic velocities above the boundary would disrupt the near-critical reflection more than the wide-angle reflection.

The CMB has a thermal boundary layer (TBL) with a temperature contrast of 800–2,000 K (refs 16, 18). The TBL does not explain our observation, because it would lead to a slower layer at the base of the mantle, rather than the observed faster layer. The low P velocity of a TBL would affect the amplitude and timing more than the waveform of the diffracted P waves. Figure 4 shows an estimate of the amplitude of the P wave for stations near the west coast of the United States. The amplitude decay with distance is simple, without the fluctuations and diminished rate of decay predicted by numerical calculations for reduced velocity TBL structures⁶. It is therefore unlikely that our inference of a fast layer is complicated by the presence of a strong velocity gradient due to the TBL.

Several nearby areas do not show such a layer. Records of

the Chinese explosion from eastern North America do not show the distinct layer arrival visible in the west. A large-aperture array study of core reflections from earthquakes beneath the Andreanof Islands² found no suggestion of short-period S-to-P conversions greater than 0.5% in or above D'' in a location about 1,500 km from the current study region. No secondary arrival or kink in the travel times as a function of distance is seen across the Washington and Northern California seismic networks from a Soviet test (50° N, 42° E, 17 December 1988). Finally, the lack of a secondary arrival closer than the distance of the travel-time kink in Fig. 3 suggests that the layering is also less pronounced at distances slightly closer to the Chinese explosion. These areas are shown in Fig. 1.

A fast layer 160–200 km thick has previously been inferred beneath the northern Soviet Union, Indonesia and the Caribbean from P-wave travel times and waveforms in the distance range from 75° to 92° (refs 8–10). These observations, although limited in array aperture and clarity of the secondary arrival, show similar features. The lowermost mantle beneath northern Alaska, where we find the fast layer, is also known to be much faster than average from tomographic studies of long-period shear waves (G. Masters, personal communication).

The factors that perturb the shear and compressional wave velocities in the lowermost mantle are not well known; nor is the relation between D'' and the thinner anomaly seen above with compressional waves. The phase transition from sodium chloride to caesium chloride structure in oxides²⁰ and a phase transition in the stishovite component²¹ have been proposed as possible explanations of D'' structure, but the pressures of these hypothesized transitions have not been measured precisely enough to require their occurrence within the D'' layer. Anisotropy caused by shearing within a boundary layer at the base of the mantle is also a possibility.

A layer that is 25% enriched in stishovite relative to the overlying mantle and 130 km thick can explain our observations. Reactions between the core and mantle^{22,23} may be important in generating a stishovite-enriched layer. Such a layer would be 1–2% fast for P waves, and consistent with the timing, amplitude and distance range of the triplication that we observe. It may be less anomalous for S waves²⁰, explaining the absence of all but subtle S-wave wide-angle reflections⁷. Finally, the small

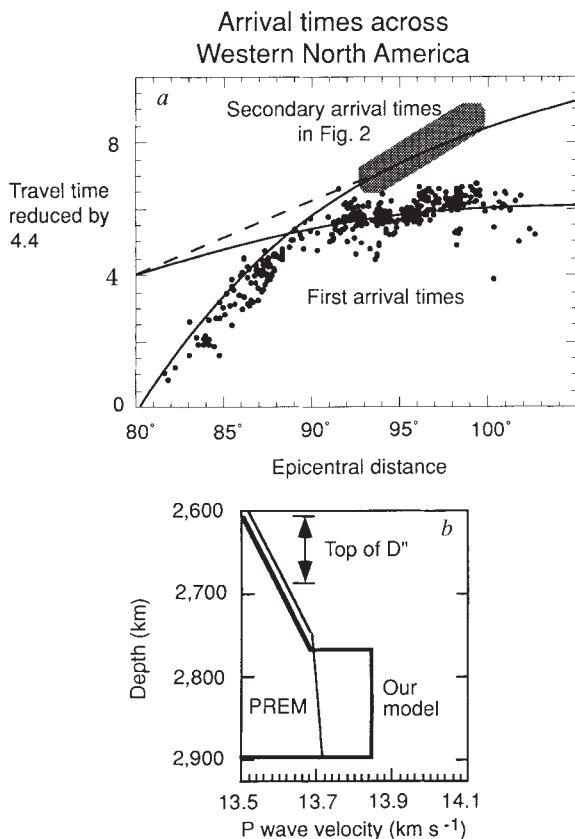


FIG. 3 a, Travel times from the Chinese nuclear test to stations in the azimuth range from 0° to 30°, which spans the western United States. The shaded region indicates approximate arrival times of the secondary arrivals identified in Fig. 2. The light line indicates the arrival times predicted by our velocity model (b), which is compared with the reference model PREM²⁵.

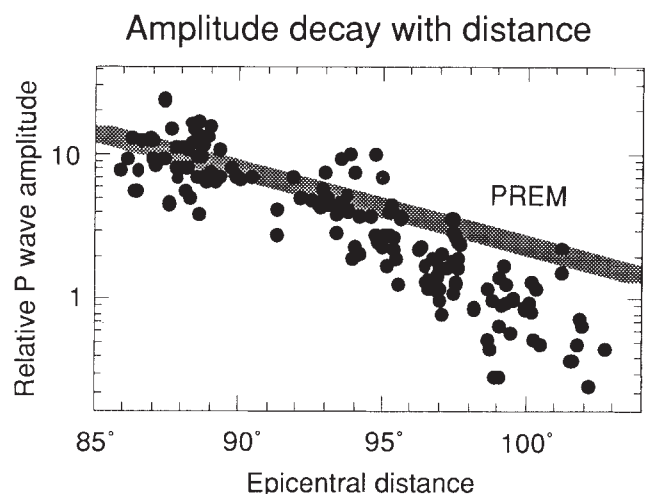


FIG. 4 The amplitude of the P arrival across the Washington, Northern California, and Southern California seismic arrays, expressed as the ratio relative to the ScP arrival from the 13 March 1992 Andreanof earthquake²⁶. ScP is expected to show slightly increasing amplitude with distance, so the P amplitudes probably decrease more rapidly with distance than shown. The ratio minimizes scatter due to near-surface impedance contrasts and focusing. The light line shows an amplitude decay curve calculated for the reference model PREM²⁵.

contrast density and, possibly, S-wave velocity or the intermittent presence of the layer would explain the lack of long-period S-wave near-vertical reflections²⁴. □

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Haldane's rule has multiple genetic causes

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HALDANE'S rule states that "When in the F_1 offspring of two different animal races one sex is absent, rare, or sterile, that sex is the heterozygous [heterogametic or XY] sex"¹. This rule represents one of the few patterns characterizing animal speciation^{2,3}. Traditional explanations of Haldane's rule^{1,4–6} claim that heterogametic hybrids are unfit because they lack an X chromosome that is 'compatible' with the autosomes of one species. Recent work^{2,7} shows that this explanation is incorrect for hybrid sterility: contrary to prediction, homogametic hybrids carrying both X chromosomes from the same species remain fertile. Until now, similar tests have not been performed for hybrid inviability. Here I show that homogametic hybrids who carry both X chromosomes from the same species are inviable. These results show that the genetic causes of Haldane's rule differ for hybrid sterility versus inviability. Haldane's rule does not, therefore, have a single genetic basis.

The cross of *Drosophila simulans* females to *D. teissieri* males obeys Haldane's rule: only hybrid females appear⁸ (Table 1). Hybrid males (the heterogametic sex in *Drosophila*) die during the first or second larval instar. The traditional Haldane–Dobzhansky–Muller^{1,4–6} explanation of Haldane's rule claims that these males are inviable because they are genetically 'unbalanced': they lack some X-linked gene product from *D. teissieri* required by the *D. teissieri* autosomes to produce fit adults. Perhaps the strongest test of this theory involves producing hybrid females who carry both X chromosomes from the same species and a haploid set of autosomes from each species (Fig. 1). Because these 'unbalanced' female hybrids also lack an X chromosome from one species they should also be unfit⁷.

Although repeated tests have disproved this explanation of Haldane's rule for hybrid sterility—unbalanced females remain fertile²—this simple test has not been performed for cases of Haldane's rule for inviability owing to the unavailability of the required genetic stocks.

As an attached-X chromosome (C(1)RM) is available in *D. simulans*, I was able to perform this critical test in the *D. simulans*–*D. teissieri* hybridization: the *D. simulans* C(1)RM female X *D. teissieri* male hybridization produces hybrid females who are just as genetically unbalanced as the inviable F_1 hybrid males. These females carry two *D. simulans* X chromosomes, a haploid set of autosomes from each species, a Y from *D. teissieri*, and cytoplasm from *D. simulans*, just as do the inviable F_1 males. As Table 1 shows, the results of this test are simple: no C(1)RM adult hybrid females ever appeared. Unbalanced females are, therefore, lethal. Sexing of hybrid larvae and pupae revealed that these females die in the same developmental stage as F_1 $X_{simulans}/Y_{teissieri}$ males (first or second larval instar).

The inviability of these females strongly suggests that the genetic basis of Haldane's rule differs for hybrid sterility and hybrid inviability. To test this possibility further, I did a similar analysis in a second hybridization. The *D. melanogaster* female X *D. simulans* male hybridization yields only females, whereas the reciprocal cross reportedly yields many males with a few rare females^{9–11}, that is, this direction of the cross is reportedly an exception to Haldane's rule. As such, this hybridization has been considered an inappropriate place to test the basis of Haldane's rule (if F_1 females are already inviable, the interpretation of any inviability of 'unbalanced' females is obviously muddled). However, we have recently discovered that many, if not all, extant strains of these species yield abundant hybrid females in both directions of the species cross, at least at lower temperatures (18°–22 °C) (see Table 1 for details). The *D. melanogaster*–*D. simulans* hybridization clearly obeys Haldane's rule.

This species pair thus affords another opportunity to test the basis of Haldane's rule for inviability. Although unbalanced hybrid females from this cross (carrying an attached X from *D. melanogaster*) have been repeatedly described as inviable^{11,12},

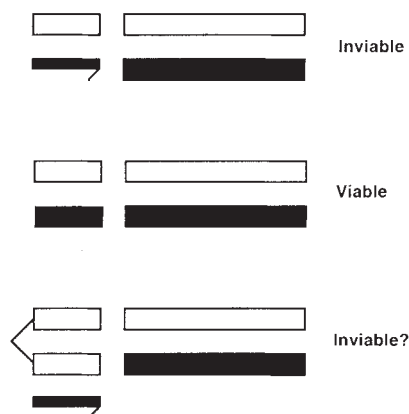


FIG. 1 Test of the genetic basis of Haldane's rule. The chromosomes of one species (for example, *D. simulans*) are shown in white and those of the other species (for example, *D. teissieri*) in black. Sex chromosomes are shown at the left (X on top; Y, with 'hook', on the bottom). Haploid sets of autosomes shown at the right. All genotypes carry cytoplasm from the 'white' species. The top genotype represents F_1 male hybrids and the middle genotype represents F_1 female hybrids. The bottom genotype depicts hybrid females who carry an attached X chromosome from the 'white' species. The traditional explanation of Haldane's rule predicts that this 'unbalanced' genotype will be inviable. (Population genetic models (H.A.O., unpublished) show that this theory requires an additional assumption: the alleles causing reproductive isolation act as loss-of-function mutations; that is more recessive alleles must have greater homozygous effects on hybrid fitness.)

TABLE 1 Results of species crosses

Hybridization	Females	Males
<i>D. simulans</i> — <i>D. teissieri</i>		
<i>D. simulans</i> Fla. City ♀ × <i>D. teissieri</i> Congo ♂	51	0
<i>D. simulans</i> yw ♀ × <i>D. teissieri</i> Congo ♂	200	0
<i>D. simulans</i> C(1)RM, yw/Y ♀ × <i>D. teissieri</i> Congo ♂	0*	0
<i>D. melanogaster</i> — <i>D. simulans</i>		
<i>D. melanogaster</i> Oregon-R ♀ × <i>D. simulans</i> v ^C ♂	87	0
<i>D. melanogaster</i> Bellows Falls ♀ × <i>D. simulans</i> v ^C ♂	46	0
<i>D. melanogaster</i> Bellows Falls ♀ × <i>D. simulans</i> Fla. City ♂	190	0
<i>D. melanogaster</i> Oregon-R ♀ × <i>D. simulans</i> vm ♂	297	0
<i>D. melanogaster</i> Napa ♀ × <i>D. simulans</i> w ^{2C} ♂	170	0
<i>D. simulans</i> v ^C ♀ × <i>D. melanogaster</i> Oregon-R ♂	83	40
<i>D. simulans</i> v ^C ♀ × <i>D. melanogaster</i> Bellows Falls ♂	176	169
<i>D. simulans</i> Fla. City ♀ × <i>D. melanogaster</i> Bellows Falls ♂	62	43
<i>D. simulans</i> vm ♀ × <i>D. melanogaster</i> Oregon-R ♂	186	471
<i>D. simulans</i> w ^{2C} ♀ × <i>D. melanogaster</i> Napa ♂	105	111
<i>D. melanogaster</i> C(1)RM, ywf/Y ♀ × <i>D. simulans</i> v ^C ♂	0	350
<i>D. melanogaster</i> C(1)RM, ywf/Y ♀ × <i>D. simulans</i> vm ♂	0	267

Number of adult hybrids appearing in species crosses. All hybrids are sterile. All markers are X-linked. Crosses performed at 22 °C and 18 °C (as results did not differ with temperature, data were pooled). In the third cross, numerous hybrid larvae and pupae appeared in ~30 vials (the *X_{teissieri}/Y_{simulans}* hybrid males survive into the pupal stage). The timing of hybrid lethality was determined by scoring the sex of hybrid larvae/pupae using the X-linked yellow marker. The reciprocal cross (*D. teissieri* female × *D. simulans* male) fails, apparently because of complete sexual isolation⁸. The appearance of abundant females in the *D. simulans* ♀ × *D. melanogaster* ♂ crosses is not due to some rare hybrid inviability rescue mutation (H.A.O. and J. Coyne, unpublished results): numerous females appear when using all tested strains of both *D. simulans* and *D. melanogaster* (at least 7 strains of each species tested). Tested strains include freshly caught wild-type flies of both species as well as older laboratory mutant and wild-type stocks (including flies from the United States, Australia and Okinawa). Although hybrid females are abundant at 18–22 °C, few appear at 25 °C.

* One hybrid female appeared who was yellow⁺, white⁺, not the expected C(1)RM, yellow, white. This female clearly resulted from the breakdown of the C(1)RM chromosome (which occasionally occurs in the *D. simulans* stock) and thus carried one X chromosome from each species.

there is little reason for believing that this remains true given the unusual present crossing behaviour of these species. I thus crossed *D. melanogaster* attached-X females to *D. simulans* males. As Table 1 shows, the resulting attached-X hybrid females remain completely inviable. Moreover, these females die during the same developmental period as hybrid males (late larval/early pupal)¹¹.

Thus, in both hybridizations, hybrid females who are homozygous for the X from one species are as inviable as hybrid F₁ males bearing the same X. These results clearly support the traditional explanations of Haldane's rule for inviability (although population genetic theory shows that these explanations require subtle modification (see Fig. 1)). Most important, these findings qualitatively differ from those obtained in analogous tests of Haldane's rule for sterility² and thus show that the genetic basis of Haldane's rule in *Drosophila* differs for inviability versus sterility, as several workers have speculated^{3,13}. Haldane's rule does not, therefore, have a single genetic cause, as it once appeared^{2,4,5}.

Nonetheless, the genetic bases of Haldane's rule for sterility versus inviability appear to differ in a logical way. In *Drosophila*, almost all mutations affecting fertility have sex-limited effects, whereas almost all mutations affecting viability do not¹⁴. Similarly, the genes causing hybrid sterility clearly have sex-limited effects: although unbalanced hybrid females are homozygous for the X-linked alleles causing hybrid male sterility, they remain fertile². Moreover, when both hybrid sexes are partially sterile, mapping experiments show that different loci affect each sex². On the other hand, the present results suggest

that the genes causing hybrid inviability affect both sexes—females made homozygous for alleles causing hybrid male inviability are lethal. Although we cannot prove that the same loci cause the lethality of attached-X hybrid females and F₁ males, this conclusion is strengthened by the finding that, in both hybridizations, these females and males die at the same time during development. Moreover, in *D. melanogaster*—*D. simulans*, the fact that the hybrid 'rescue mutation' *Hmr* restores the viability of both F₁ hybrid males and attached-X hybrid females¹¹ strongly suggests that these hybrids suffer from the same developmental problem.

In conclusion, the behaviour of the factors causing reproductive isolation between species is strikingly similar to that of alleles affecting fitness within species: factors affecting fertility have sex-limited effects, but those affecting viability apparently do not. This parallel provides the clearest evidence to date that speciation involves the divergence of 'ordinary' genes with normal functions within species¹⁵, as proposed by the founders of the modern synthesis. □

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Induction of immediate spatiotemporal changes in thalamic networks by peripheral block of ascending cutaneous information

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PERIPHERAL sensory deprivation induces reorganization within the somatosensory cortex of adult animals^{1–6}. Although most studies have focused on the somatosensory cortex^{1–6}, changes at subcortical levels (for example the thalamus) could also play a fundamental role in sensory plasticity^{7–11}. To investigate this, we made chronic simultaneous recordings of large numbers of single neurons across the ventral posterior medial thalamus (VPM) in adult rats. This allowed a continuous and quantitative evaluation of the receptive fields of the same sample of single VPM neurons per animal, before and after sensory deprivation. Local anaesthesia in the face induced an immediate and reversible reorganization of a large portion of the VPM map. This differentially affected the short latency (4–6 ms) responses (SLRs) and long latency

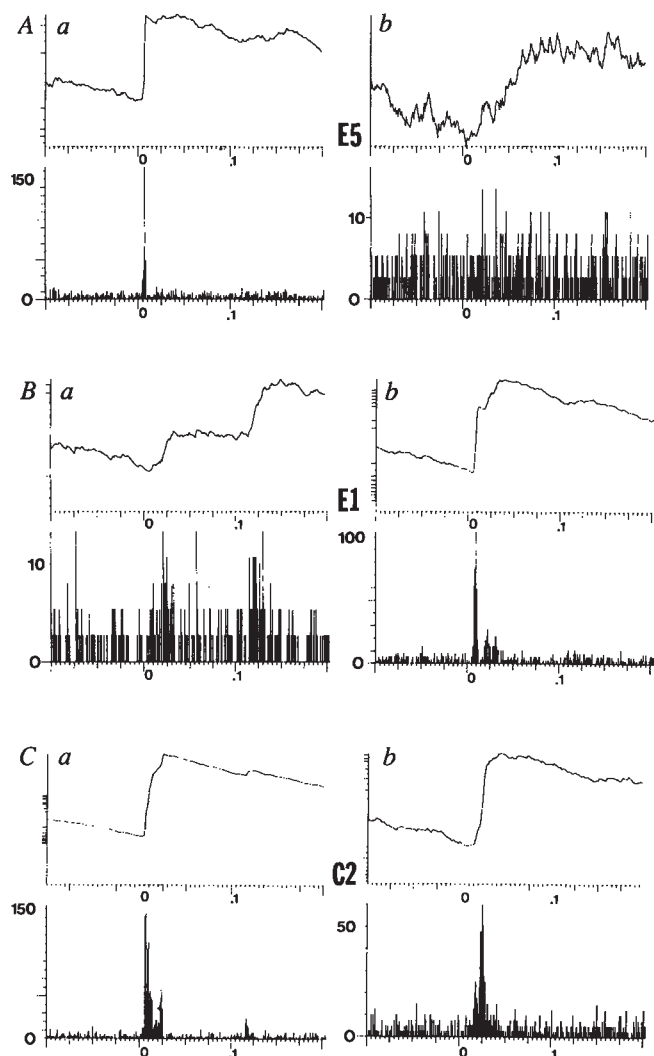


FIG. 1 Local anaesthesia of the rostral face alters the sensory responses of three simultaneously recorded neurons in the VPM of a pentobarbital anaesthetized rat. For each cell, cumulative frequency histograms (CFHs; above) and post-stimulus time histograms (PSTHs; below) show the magnitude and timing of different latency responses of these neurons to 360 single stimulations of different whiskers (E5, E1 and C2) before (Aa–Ca) and after (Ab–Cb) subcutaneous lidocaine injection in the maxillary gum. Response magnitudes, in average instantaneous firing rate per bin (in spikes per s) are shown on the vertical axes of PSTHs. The scales are different for each PSTH to highlight the effects. The vertical dimension in CFHs is used to plot the deviation of the cumulative firing above or below the average firing rate. Scales on the vertical axes of CFHs show negative log P -values (for example at the first scale mark $P=0.1$, at the second, $P=0.01$ and so on). These show the probability that the overall distribution of cumulative frequencies differs from a random distribution, as computed using a one-way Kolmogorov-Smirnov test. Thus, the highly significant response in A ($P<10^{-5}$) became insignificant ($P>0.1$) after the lidocaine injection. Horizontal axis: pre- and post-stimulus time in s; whisker deflection up at 0, down at 0.1 s; bins=1 ms, scale marks=5, 25 and 100 ms.

METHODS. Experiments were done on six adult Long-Evans (hooded) rats. One week before experimentation bundles of 8–16 teflon-coated stainless steel 25 or 50 μ m tip microwires were surgically implanted in the VPM thalamic nucleus. Up to 23 single VPM neuronal signals were simultaneously amplified, filtered and discriminated using an apparatus from Spectrum Scientific (Dallas, TX). Each single extracellular spike waveform was time-voltage window discriminated, and statistically validated using principal components analysis. This ensured that the same set of single neurons was recorded throughout each experiment. Cutaneous sites were stimulated at a rate of 1 Hz, using a computer-controlled vibromechanical probe delivering 100 ms duration step pulse displacements (whiskers were moved 3°; furry skin was depressed 0.5 mm). For each experiment, 11–20 single whiskers or skin sites were independently stimulated in a random sequence.

(15–25 ms) responses (LLRs) of single VPM neurons. The SLRs and LLRs normally define spatiotemporally complex receptive fields in the VPM¹². Here we report that 73% of single neurons whose original receptive fields included the anaesthetized zone showed immediate unmasking of SLRs in response to stimulation of adjacent cutaneous regions, and/or loss of SLRs with preservation or enhancement of LLRs in response to stimulation of regions just surrounding the anaesthetized zone. This thalamic reorganization demonstrates that peripheral sensory deprivation may induce immediate plastic changes at multiple levels of the somatosensory system. Further, its spatiotemporally complex character suggests a disruption of the normal dynamic equilibrium between multiple ascending and descending influences on the VPM.

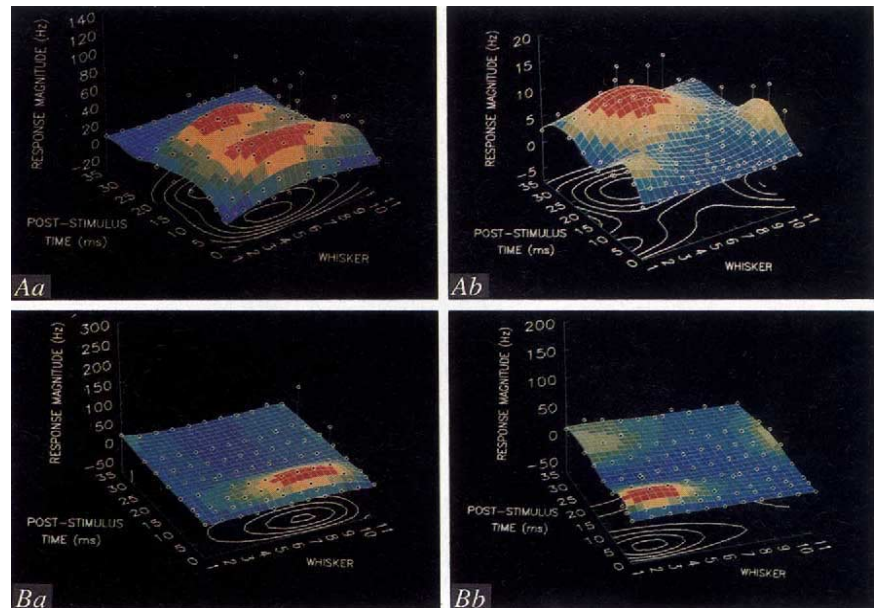
To investigate whether peripheral deprivation produces immediate reorganization of sensory maps in subcortical structures such as the thalamus, we developed neurophysiological techniques allowing chronic simultaneous recordings of large numbers of single neurons distributed across topographic representations in sensory nuclei. This approach overcame many limitations of the classic mapping technique because it allowed: (1) the same neurons to be recorded before and after sensory deprivation, (2) a precise quantitative definition of their spatiotemporal response properties, and (3) a simultaneous sampling of short- and long-term neuronal changes occurring across the whole sensory representation embedded in the nucleus. This new approach was used to demonstrate that the map of the face in the VPM thalamus of adult rats is immediately reorganized following reversible peripheral sensory deprivation induced by small (0.01–0.04 ml) subcutaneous injections of lidocaine (1%). This reorganization was manifested by marked changes in the spatiotemporal response properties of single VPM neurons to stimulation of facial locations within the fully and partially anaesthetized zones, and also their immediate surrounds.

Figure 1 illustrates typical spatiotemporal modifications observed within an ensemble of single thalamic neurons following a small (0.04 ml) injection of lidocaine in the gum just behind the maxillary incisors in a pentobarbital anaesthetized (50 mg kg^{-1} intraperitoneally) adult rat. Before this injection, most of the total 11 neurons that were simultaneously recorded in this rat responded robustly to computer controlled vibromechanical stimulation of up to 16 whiskers across the face, including whisker E5 (as in Fig. 1Aa). After the injection, these responses to E5 were completely blocked (Fig. 1Ab), thus defining this rostral whisker as part of the zone anaesthetized by the lidocaine spread. By contrast, stimulation of more caudally placed whiskers (for example, whisker E1) yielded strong short latency (5–10 ms) responses in many neurons that had previously been unresponsive at that latency (Fig. 1Ba, b). Such complementary changes in response magnitudes produced a spatial shift of single neuronal receptive fields away from the anaesthetized zone towards surrounding unanaesthetized regions.

Beyond these changes in the spatial domain, the focal anaesthesia also altered the normal latency distribution of sensory responses of 10 neurons within the ensemble. For example, the neuron in Fig. 1Ca normally responded at both short (5–10 ms) and long (15–25 ms) latencies to stimulation of whisker C2. Lidocaine selectively blocked the short latency response, thus shifting the temporal profile of the post-stimulus time histograms (see Fig. 1Cb).

The statistical significance of the sensory-evoked responses in these neurons was routinely assessed using: (1) the Kolmogorov-Smirnov (KS) test, which estimates the deviation from randomness of the cumulative frequency distribution, and (2) analysis of variance (ANOVA), which determines whether sensory-evoked responses in specific time epochs are significantly different from spontaneous discharge. All of the effects reported here were found to be highly significant ($P<0.01$). The deprivation-induced changes occurred immediately (within about 3 minutes) after the lidocaine injection and lasted 4–6 hours. In all cases in which the neurons were re-recorded 12–24

FIG. 2 Spatiotemporal receptive fields of VPM neurons are altered by lidocaine anaesthesia of the rostral face. The colour-coded three-dimensional surfaces here define the spatiotemporal receptive fields of two VPM neurons before (*Aa* and *Ba*) and after (*Ab* and *Bb*) a lidocaine injection in the maxillary gum of an adult rat. These were constructed by sequentially stimulating 11 whiskers 360 times apiece, and measuring average instantaneous evoked firing rate during each of eight different post-stimulus time epochs (shown on the left horizontal axis, ranging from 0 to 35 ms). The responses for each stimulated whisker were rank ordered according to their relative caudal-to-rostral position in the whisker pad (ordered from 1–11 as follows along the right horizontal axis: B1, E1, E2, C1, C2, D2, E3, C3, E4, E5 and E6). Response magnitudes are shown in Hz (spikes per s) on the vertical axis. The range of response magnitudes was coded by eight colours, in which red represents the greatest firing rate and dark blue the lowest. All colour codes were automatically scaled according to the vertical range, and thus vary from graph to graph. Actual data points are represented by white open circles whose distances from the spline smoothed surface are indicated by vertical dotted lines. All of the major topographical features shown here were shown to be significant ($P < 0.01$) using the ANOVA and KS tests.



hours after the lidocaine injections their receptive fields were found to be similar to pre-lidocaine controls.

The major conclusion to be derived from the above analyses of PSTHs is that peripheral-sensory deprivation disrupts both spatial and temporal features of thalamic neuronal responses. To visualize how the responses seen in PSTHs (as in Fig. 1) contribute to the definition of single cell receptive fields, we used three-dimensional computer graphics to construct 'spatiotemporal receptive fields', which depict neuronal response magnitude (in spikes per s) as a function of stimulus position and post-stimulus time. For this, quantitative measurements from the PSTHs were used to generate a three-dimensional surface, which was smoothed using a spline algorithm based on a moving third-order polynomial function. To reduce the impact of statistical outliers, a low 'stiffness' coefficient (0.15) was used.

Figure 2 shows spatiotemporal receptive fields of two typical neurons before (Fig. 2*Aa*, *Ba*) and after (Fig. 2*Ab*, *Bb*) the same lidocaine injection as in Fig. 1. These quantitative representations revealed that receptive fields in the VPM are much larger and spatiotemporally complex¹² than reported^{13,15}. Lidocaine induced two general categories of receptive field reorganization in the VPM: (1) temporal shifts (as in Fig. 2*Aa*, *b*) and (2) spatial shifts (as in Fig. 2*Ba*, *b*). Figure 2*Aa* depicts a receptive field that covers a wide area of the whisker pad during both short and long latency post-stimulus time epochs. After the lidocaine injection, this receptive field changed dramatically (Fig. 2*Ab*). At short latencies, the centre of the receptive field disappeared, while its surround was enhanced at both edges. By contrast, the long latency peak remained almost intact, though somewhat prolonged. Thus, the main effect of the deprivation was to shift temporally the main response of this neuron from short to long latency epochs.

By contrast, Fig. 2*Ba*, *b* shows an example of a predominantly spatial shift. Lidocaine-induced sensory deprivation produced a marked spatial shift in the short latency receptive field peak of this neuron from its original location in the rostral whiskers (C3 and E3–E5) to the caudal whiskers (B1, C1 and E1–E2). In addition, two weak long latency response peaks emerged. Similar patterns of reorganization were observed in each of eight equivalent experiments, involving chronic recordings from a

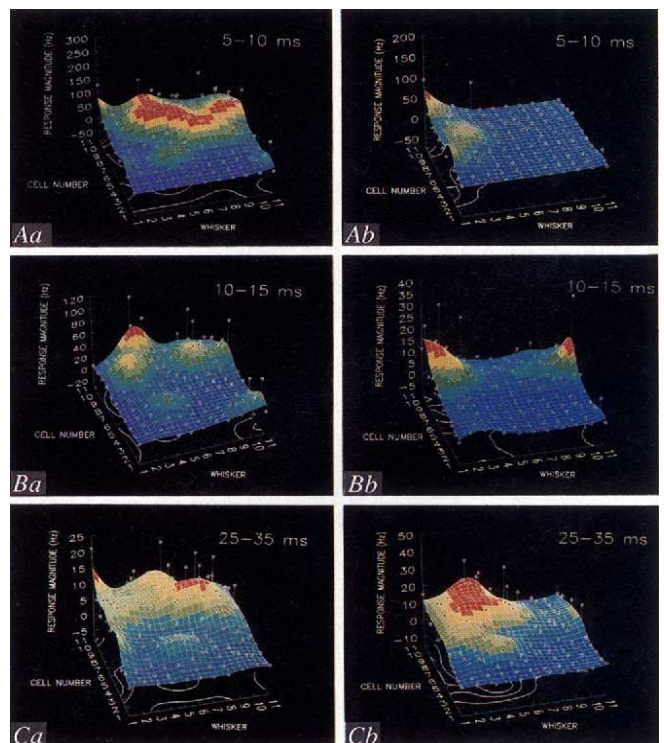


FIG. 3 Population receptive fields of VPM neuronal ensembles are altered by lidocaine anaesthesia in the rostral face. Each colour-coded three-dimensional surface here represents the sensory-evoked responses of an ensemble of 11 simultaneously recorded VPM neurons to stimulation of each of 11 whiskers (same as in Fig. 2) within each of three post-stimulus time epochs (5–10, 10–15 and 25–35 ms). *Aa*–*Ca* show the population receptive fields recorded during control. *Ab*–*Cb* show the same population receptive fields following the lidocaine injection. The vertical axis, right horizontal axis, and the colour coding of the three-dimensional surfaces are the same as in Fig. 2. The 11 neurons that comprise the left horizontal axis were rank ordered according to the relative position of their receptive field centres and their overall sensory responsiveness.

total of 75 VPM neurons in six rats. Of the 75 neurons, 55 had receptive fields within the anaesthetized zone and/or its immediate surround. Of these, 30 cells (55%) exhibited the temporal shifts (category 1, as in Fig. 2*Aa, b*), and 27 cells (49%) exhibited the spatial shifts (category 2, as in Fig. 2*Ba, b*). These effects were partially overlapped, such that both were observed in 17 cells (31%). Finally, 11 cells did not change, and 4 cells were completely anaesthetized.

The next step was to determine how the spatiotemporal complexity of the lidocaine effects on the receptive fields of single neurons was translated into changes in the sensory map embedded in the neuronal population. Three-dimensional graphics were used to construct population receptive fields, which depict, within given post-stimulus time epochs, the magnitudes of sensory responses across the population of recorded neurons as a function of stimulus location. The population receptive fields in Fig. 3*Aa–Ca* show how the spatiotemporal receptive fields of 11 simultaneously recorded single neurons were integrated into a sensory map which dynamically changes over post-stimulus time. The lidocaine produced the alterations shown in Fig. 3*Ab–Cb* by creating an anaesthetized zone in which short latency responses to stimulation of whiskers C3 and E3–E5 (7–10 in Fig. 3*Ab* and *Bb*) were blocked. This induced a spatial shift of the short latency responses toward 'far surround' whiskers B1, E1, and E6 (1, 2 and 11 in Fig. 3*Bb*), distal to the anaesthetized zone. Furthermore, long latency responses tended to remain, or newly appear, in 'near surround' whiskers E2, C1 and 2, and D2 (3–6 in Fig. 3*Cb*), more proximal to the anaesthetized zone. The net effect was to increase the relative strength of the longer latency responses which partially 'filled in' the area vacated by the short latency responses, conceivably providing a 'seed' for longer term reorganization.

These results demonstrate that local peripheral sensory deprivation induces, within minutes, a marked reorganization of the somatosensory map in the VPM thalamus. The purely spatial aspects of these receptive field shifts are reminiscent of the spatial reorganizations of the sensory map in the somatosensory cortex which were defined using classic mapping studies^{1–3}. Moreover, they are quite similar to the immediate unmasking of cortical neuronal responsiveness observed after similar sensory deprivation models^{4–6}. The results here, therefore, show that the short-term plastic reorganization in the somatosensory system is not restricted to the cerebral cortex, and provide direct quantitative evidence for previous suggestions that the thalamic map may be reorganized after different types of sensory deprivation^{7–11,14,15}. Thus, the long-term reorganizations observed in the cortex could derive at least partially from changes at lower levels such as the thalamus.

Another important issue raised by the quantitative approach used here is that thalamic receptive fields, which normally exhibit complex spatiotemporal dynamics¹², are altered by peripheral deafferentation. This spatiotemporal complexity might be attributed to the asynchronous convergence of multiple feedforward^{15–16} and feedback¹⁷ excitatory inputs to the VPM, which could be the sources of the different latency response components observed in these neurons. This is supported by our observations that short and long latency responses in the VPM have different receptive fields¹², and further that lidocaine affects them selectively. The lidocaine-induced unmasking of these various components could result from changes in the tonic activity of inhibitory neurons acting on the VPM or trigeminal nuclei. As a hypothesis, therefore, we suggest that sensory deprivation disrupts the normal dynamic state of equilibrium between excitation and inhibition within the network that comprises the entire somatosensory system, producing reorganizations at multiple levels of this pathway. As such, the population receptive fields in Fig. 3*Ab–Cb* could be considered as snapshots of the new dynamic equilibrium states of the somatosensory network as viewed at the thalamic level. This theoretical framework for a network-based model of sensory deprivation-induced

plasticity might reasonably be applied to other systems of the brain. □

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Cloning and expression of an adenylyl cyclase localized to the corpus striatum

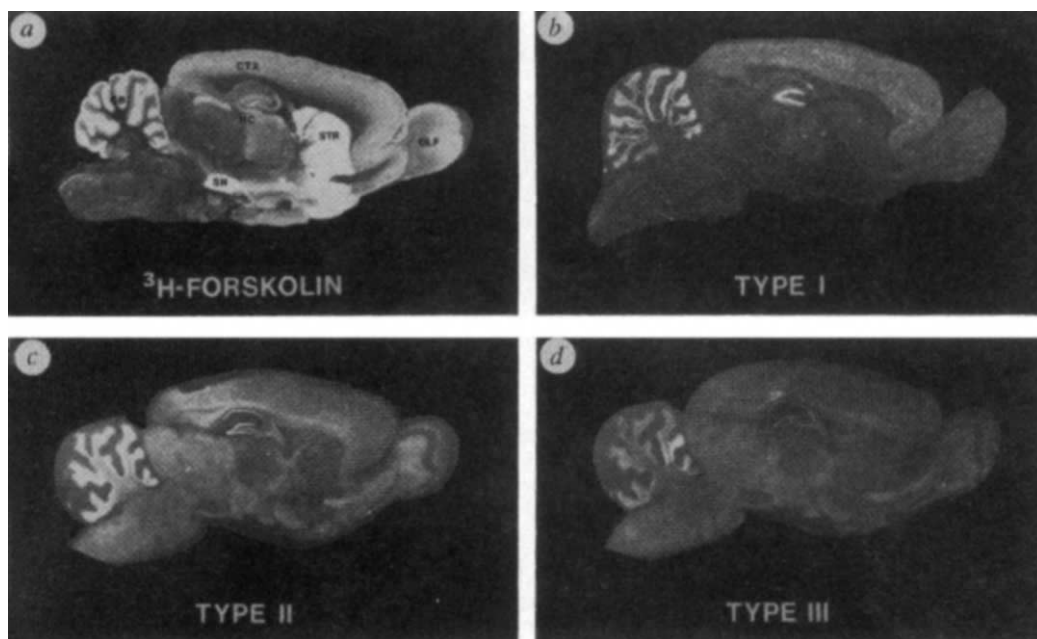
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THE neurotransmitter dopamine acts through various receptor subtypes that are largely associated with enhancement or inhibition of adenylyl cyclases^{1,2}. These dopamine-sensitive adenylyl cyclases are highly concentrated in the corpus striatum and associated limbic structures of the brain, where their levels exceed by orders of magnitude^{3,4} those in other areas of the brain. Here we use *in situ* hybridization to show that messenger RNA for three of these adenylyl cyclases^{5–7} is not found in the corpus striatum. We have isolated and expressed a complementary DNA encoding new adenylyl cyclase whose selective concentration in the corpus striatum indicates that it may be responsible for the synaptic actions of dopamine.

Binding of [³H] forskolin to adenylyl cyclase is highly concentrated in the corpus striatum (ST), the related limbic areas, and the substantia nigra to which ST fibres project^{3,4} (Fig. 1). Forskolin can interact with glucose transporters⁸, but cytochalasin B, which potentially competes with forskolin at the glucose transporter, does not change the pattern of [³H]forskolin autoradiography in the brain (data not shown). *In situ* hybridization reveals no evidence for concentration in ST of mRNA for type I, type II or type III adenylyl cyclase. The type I enzyme is most concentrated in granule cells of the cerebellum and the dentate gyrus of the hippocampus, as found previously⁹. Type II is less abundant than type I, with some concentration in granule cells of the cerebellum, CA-1 and CA-4 of the hippocampus, as well as in granule cells of the dentate gyrus. Type III adenylyl cyclase is reported to be selectively localized to olfactory neurons⁷, but we observe specific mRNA signals for type III that are more homogeneously distributed throughout the brain than type I or type II adenylyl cyclases, with some concentration in the granule cells of the cerebellum and throughout the hippocampus. No concentration in the ST is apparent for types I, II or III enzymes; type IV is found in rat brain in very small amounts¹⁰. Perhaps because of this scarcity, we could not detect type IV mRNA by *in situ* hybridization (data not shown). Sense-strand controls showed that nonspecific hybridization was low in all experiments (data not shown).

FIG. 1 Localizations of adenylyl cyclase protein and mRNA for individual cloned isoforms. *a*, [3 H]forskolin autoradiography in a sagittal section of rat brain. Highest levels of binding are seen in the striatum (STR) and substantia nigra (SN). Other areas of binding include the granule cell layer of the cerebellum (CB), cerebral cortex (CTX), hippocampus (HC), and olfactory bulb (OLF). *b*, *In situ* hybridization to type I adenylyl cyclase mRNA. High levels of expression are seen in the granule cells of the cerebellum and dentate gyrus of the hippocampus, with some grain density in the cerebral cortex. *c*, *In situ* hybridization to type II adenylyl cyclase mRNA. High expression is seen in CA-1, CA-4 and dentate gyrus of the hippocampus. Other areas of high grain density are the granule cells of the cerebellum, olfactory bulb, cerebral cortex, superior and inferior colliculi, as well as the brain stem. *d*, *In situ* hybridization to type III adenylyl cyclase mRNA. Overall expression is lower and more homogeneous than that for types I and II. Expression is seen throughout the hippocampal formation as well as in the granule cells of the cerebellum, cerebral cortex and olfactory bulb.



METHODS. [3 H]forskolin autoradiography was done as described³ with the addition of 5 μ M cytochalasin B to all incubation solutions to prevent binding to brain glucose transporters. *In situ* hybridization was as described¹⁵, with a pool of three end-labelled 45-base oligonucleotides complementary to the 5' and 3' untranslated regions of the individual cDNAs for types II and III. A pool of two probes was used for type I. Specific probes sequences were:

type I, CGAGTGGCGTGATCCGCTCTGGGCTGGGTG, GGTCTACTTCAGTACGCT-CAGCCACGGATGT; type II, ACGGTATTTCCAGAGAAGAATCGACCCAGAAC-ATAATATACAC, AGAAGCTGAGAGTCTCAGCTGAGCCCCAAGGACCGACCACT-GGC, ATATGCAAACTCCACCATGGTCTATGTGCATGTACTGACGCTC; type III, ATGTCTCCCTCCAGTGTGGACCGCTGTGGAGCCAGGGCTGTTCA, CAGCCACA-CAGGAGGCTGGAGGAAATCTGAAACCTAAAGTCTAT, TTTCAAGATTATCAG-GAACATCGGGTAAGGGCCATTGACTTAAGT; type IV, CGGAGACCCCTGCCTG-TTGTGTGAAGCTCCTGAAGTCTCGTAG, CTGCAGATGCCTGGAGACATTTT-ATTGAGGATCTTGAGAAGGGG, AGGTGTAGATGGTATAGATGAAAGAACACGG-TCCACCATGATGCCCT.

To search for an ST-specific adenylyl cyclase, we used a mixture of full-length cDNAs encoding types I, II and III enzyme to screen a corpus striatum cDNA library at low stringency. We isolated a full-length cDNA whose sequence was different from that of any previously reported adenylyl cyclase (Fig. 2). The deduced amino-acid sequence of this new enzyme, designated AC_{ST}, resembles that of other isoforms. The primary sequence has an internal homology that divides the protein into two halves. Hydropathy analysis predicts two sets of six transmembrane domains, each followed by a hydrophilic putative catalytic domain. Sequence comparison of AC_{ST} with its isoforms shows ~80% homology in the catalytic domains and low homology in the transmembrane domains.

Northern blot analysis of various brain regions and peripheral tissues revealed hybridization only in the ST; there are two mRNA species of ~6 and 7.5 kilobases (kb) (Fig. 3a). Poly(A)⁺ selection to remove distortion from ribosomal RNA resolved the doublet into four discrete bands (Fig. 3b). *In situ* hybridization shows that AC_{ST} is concentrated in the ST and related regions (Fig. 3c). Silver-grain density is very high and uniform throughout the caudate-putamen, with comparable intensity in the nucleus accumbens and olfactory tubercle. Even after prolonged exposure, we do not detect AC_{ST} in any other brain structures. Sense-strand controls showed that nonspecific hybridization was low (data not shown).

The cDNAs have recently been cloned for an adenylyl cyclase from dog heart¹¹, which shares 95% sequence homology with AC_{ST}, and for an adenylyl cyclase from rat kidney, whose sequence is identical to AC_{ST} (ref. 12): these have both been designated AC type V (refs 11, 12). Our *in situ* hybridization studies reveal small amounts of AC_{ST} in rat heart and kidney, predominantly in blood vessels; this is in accord with the low

levels of vascular dopamine receptors in these tissues (manuscript in preparation).

To establish that AC_{ST} is an active enzyme, we transfected human kidney 293 cells with the cDNA inserted into an expression vector and assayed for adenylyl cyclase activity (Fig. 4). The cells have an endogenous activity which is stimulated by forskolin and, to a lesser extent, by GTP- γ S. Cells transfected with AC_{ST} show increased basal activity and stimulation by

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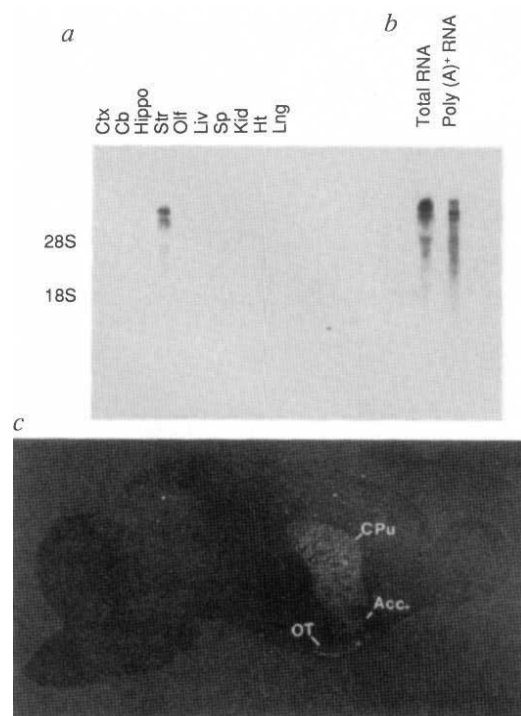
1 MATPTPPGDR PAAPPSDLGE PVTQQQQRL ASRSRGDDDD EDPPLSGDDP LEASASASVL
61 SRPGRSAAAT TAVAAAGGSG GARIAGGGST RAPLRAAVAV RRRPQQPQVA RRCAPARWRW
121 AWRSEVEKAE RPREELEPGTG TVEDGGDGED GSSSVASGSG GTTVLSLGAC CLALLQIFRS
181 KKFPSDKLER LYQRYFFRLN QSSLTMLMAE LVLVCLVMA FAARPLQV VYLAVLAPAV
241 GVILIMAVLC NRAAFHQDHM GLACYALIAV VLAVGVVGLL LPQPSASEG IWWTVFFIYT
301 IYTLFVRMR AAVLSGVLLS ALHLAISLHT NAQDQFLKQ LVSNVLTFSQ TNIVGVCTHY
361 PAEVSQORAF QETRECIQAR LHSQRENQQQ ERLLSLVLR HVAMEMKADI NAKQEDMMFH
421 KIYIQKHNV SILFADIEGF TSLASQCTAQ ELVMTLNELF ARFQDLAAEN HCLRIKILGD
481 CYVCVSGLPE ARADHAHCCV EMGMDMIEAI SLVREVTVGN VNMVGVHSG RVHCGVLGLR
541 KWQFDVWSSD VTLANHMEAG GKAGRIHITK ATNLNLYGNDY EVEPGCGGER NAYLKEHSIE
601 TFLILRCTQK RKEEKAMIAK MNRQRTNSIG HNPFWGAER PFYNHLLGGNQ VCKEMKRMGF
661 EDPKDKNAQE SANPEDEVDE FLGRAIDARS IDRLRSEHVR KFLTLTFREP LEKKYSKQVD
721 DRFGAYVACA SLVFLFICFV QITVPHSIF MSLFVLSCLF LLALVVFISV IYACVKLFPT
781 PLQTLRSRIV RSKKNSTLVG VFTITLVFLS ALGNMFMCNS KNLVGLCLAGE HMITVNVQNA
841 CHVMESGFNY RLGDQGGFCG SPQSNCFNFE YFTYVLLSL LACSFLQLIS CIGKVLMLIA
901 TELIYVLIGE VPGVTLFDNA DLLVTANAIQ FRNNGTSQCF EHATKVALKV VTPIIISVVF
961 LALYLHAQVQ ESTARLDFLW KIQATEEKEE MEELQAYNR LHLNLPKDV AAHFLARERR
1021 NDLEYVQSCQ CVAVMFASIA NFSEFVLELE ANNEGVCELR LLNEIADFD EIISEDRFRQ
1081 LEIKITIGST YMAASGLNDS TYDKAGKTHI KALADFAMKL MDQMKYINEH SFNNFMQKIT
1141 LNIQPVVAVG IGARKPYQDI WGNVTNVASR MDSTGVDPRI QVTTDMYQVL AANTYQLECR
1201 GVVKVKGGE MMTYFLNGGP PLS

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FIG. 2 Amino-acid sequence of AC_{ST} (single-letter amino-acid notation).

METHODS. A rat striatal cDNA library (Clontech) was screened in triplicate with a mixture of random-primer-labelled full-length cDNA clones of the rat type II and III and bovine type I adenylyl cyclases. Duplicate filters were washed at low stringency (55 °C, 2 $\times$ SSC); the final filter was washed at high stringency (65 °C, 0.5 $\times$ SSC). Plaques that hybridized in duplicate at low stringency but not at high stringency were isolated and sequenced (Sequenase, USB). Novel clones were used in a second round of screening at high stringency to isolate a full-length cDNA clone.

FIG. 3 AC_{ST} expression in brain regions and peripheral tissues. *a*, Northern blot analysis of total RNA (20 mg) from cortex (Ctx), cerebellum (Cb), hippocampus (Hippo), striatum (Str), olfactory bulb (Olf), liver (liv), spleen (Sp), kidney (Kid), heart (Ht), and lung (Lng). AC_{ST} is seen only in the striatum where two mRNA species are apparently recognized. Levels of total RNA loaded in each lane were equal as verified by ethidium bromide staining of the gel as well as reprobing of the blot with a beta actin probe (data not shown). *b*, Poly(A)⁺ selection of striatal RNA resolves doublet into four apparent RNA species. *c*, *In situ* hybridization to AC_{ST} mRNA in a sagittal section of rat brain. Expression is limited to dopamine-containing striatal and mesolimbic structures. Grain densities are shown in the caudate/putamen (CPu), nucleus accumbens (Acc.), and olfactory tubercle (OT). METHODS. RNA was purified by tissue homogenization in guanidine isothiocyanate followed by CsCl gradient centrifugation¹⁶. RNA was separated on a formaldehyde-agarose gel, transferred to a nitrocellulose filter and probed with a random-primer-labelled probe representing the 3'-most 1 kb of the full-length cDNA. The filter was then washed at high stringency (65 °C, 0.5× SSC). *In situ* hybridization was as described for Fig. 1 with a mixture of three oligonucleotide probes directed against the AC_{ST} cDNA. Specific probe sequences were: GTTCGAGAGCACAGCCATGATAAGGATCAC-GCCACAGCAGCTGC, CCCTCCTGTTGCTGGTCTCCCGCTGTGAGTGGAGCCGAG-GCTTGG, TGGATGCCAGGCTAGTGAAGCCTTCATGTCAGCAAACAGATGC.



GTP- γ S and forskolin which is sixfold greater than endogenous levels; this is comparable to the enhancement of forskolin-stimulated activity observed with transfected types I–IV adenylyl cyclase^{5–7,10}.

The markedly selective localization of AC_{ST} in the corpus striatum and related structures has several implications. For reasons that are not immediately evident, certain signal transduction systems have isoforms that operate selectively in the

ST, presumably in conjunction with dopamine neurotransmission. The G protein G_{olf} is localized to ST, but no other G protein α -subunits are present in significant amounts in ST^{13,14}. This suggests that dopamine neurotransmission is uniquely coupled to G_{olf} and AC_{ST} , and it will be interesting to see whether AC_{ST} couples more efficiently to G_{olf} than to other G proteins.

The identification of AC_{ST} may be important in drug design. The principal antipsychotic neuroleptics block dopamine receptors, whereas agonists at dopamine receptors have anti-parkinsonian actions. No therapeutic agents have been developed that stimulate or block adenylyl cyclase because this enzyme is so ubiquitous that such drugs would exert many side effects. The localization of AC_{ST} to the brain and its absence from peripheral organs suggest that AC_{ST} agonists or antagonists could have therapeutic potential in neurological and psychiatric disorders. □

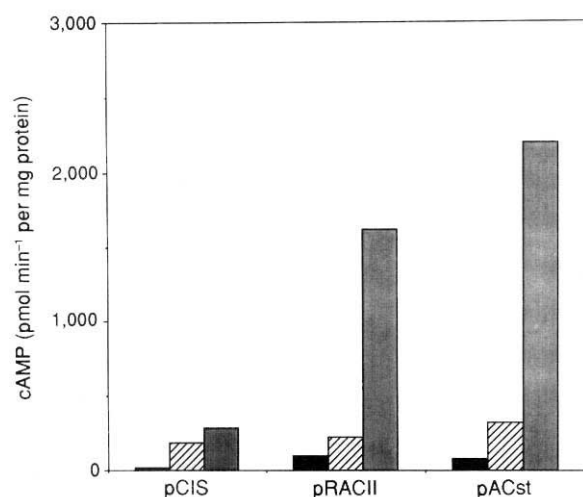


FIG. 4 Functional expression of AC_{ST} enzyme activity. *a*, AC activity was measured in kidney cells transfected with the control plasmid (pCIS), type II adenylyl cyclase (pRACII), or AC_{ST} (pACst). Activity was measured under basal conditions (solid bars), 100 mM GTP- γ S-stimulated (striped bars), and 100 mM forskolin-stimulated (dotted bars).

METHODS. Full-length cDNA was excised from Bluescript (Stratagene) with *Xho*I and inserted into the expression vector pCIS-2 (ref. 17). Transient transfection was done by calcium phosphate precipitation as described¹⁸. Cells were collected 3 d after transfection and homogenized in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 1 mM EDTA, 1 mM phenylmethylsulphonylfluoride (PMSF), and 1 mg ml⁻¹ leupeptin. Enzyme activity was assayed as described⁷.

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Defective expression of T-cell CD40 ligand causes X-linked immunodeficiency with hyper-IgM

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X CHROMOSOME-LINKED immunodeficiency with hyper-IgM (HIGM1, MIM number 308230) is a rare disorder characterized by recurrent bacterial infections, very low or absent IgG, IgA and IgE, and normal to increased IgM and IgD serum levels¹⁻³. HIGM1 has been suggested to result from ineffective T-cell help for B cells⁴. We and others⁵⁻⁸ have identified a novel, TNF-related activation protein (TRAP) that is exclusively expressed on the surface of stimulated T cells^{6,8}. TRAP, a type II transmembrane protein of *M_r* 33,000, is the physiological ligand for CD40 (refs 5-8). Crosslinking of CD40 on B cells induces, in the presence of lymphokines, immunoglobulin class switching from IgM to IgG, IgA or IgE^{5,9-11}. Mapping of the TRAP gene to the X-chromosomal location q26.3-q27.1 (ref. 6) suggested a causal relationship to HIGM1, which had previously been assigned to Xq26 (refs 12-14). Here we present evidence that point mutations in the TRAP gene give rise to nonfunctional or defective expression of TRAP on the surface of T cells in patients with HIGM1. The resultant failure of TRAP to interact with CD40 on functionally intact B cells is responsible for the observed immunoglobulin isotype defect in HIGM1.

We studied three affected males (T.G., B.W., A.T.) from unrelated families (TU, WG, HB, respectively) in which inheritance of HIGM1 could clearly be mapped to q26 region of the

X chromosome^{13,14}. We first analysed the patients' T cells for surface expression of TRAP after activating unseparated peripheral blood lymphocytes (PBL) by a combination of the phorbol ester phorbol myristate acetate (PMA) and the Ca^{2+} -ionophore A23187, the most potent stimulation mode (our unpublished observations). Because TRAP is exclusively expressed on activated T cells and not on B cells or monocytes^{6,8}, isolation of T cells before analysis was unnecessary. Functional expression of TRAP was determined using a soluble CD40-Ig chimaeric molecule in immunofluorescence flow cytometry. Expression was further confirmed by positive staining with TRAP-specific rabbit antiserum generated against a recombinant polypeptide encompassing nine amino-acid residues of the transmembrane region and the first 137 amino acids of the extracellular portion of the TRAP protein (compare with Fig. 2). Activated T cells from healthy controls express significant amounts of TRAP on the cell surface which can be detected with either CD40-Ig or anti-TRAP serum, whereas unstimulated T cells do not express TRAP (Fig. 1). When we analysed activated PBL from T.G. no staining could be detected with CD40-Ig; however, a clear signal was obtained with anti-TRAP serum, indicating either partial expression of the TRAP molecule on the cell surface or expression of a conformationally altered protein (Fig. 1). Similar results were observed with activated T cells from B.W. (Fig. 1). Flow cytometry profiles obtained with activated T cells from A.T. were different in that there was no staining with either CD40-Ig or anti-TRAP serum (Fig. 1). In all experiments, proper stimulation conditions were controlled by including PBL from healthy individuals as positive controls, as well as by analysing cell surface expression of CD69, a molecule that is upregulated early after T-cell activation (data not shown). Expression levels of CD40, the molecule interacting with TRAP, were comparable to healthy controls in all three patients (data not shown).

To identify the basis for the failure of the patients' T cells to bind CD40, TRAP messenger RNA was investigated. Northern analysis done with PBL from T.G. and A.T. activated by PMA and Ca^{2+} -ionophore for 4 h gave results comparable to normal controls, thus excluding gross abnormalities of gene transcription or RNA splicing in these patients (data not shown; not enough cell material was available for B.W.). Subsequently, RNA from all three patients was reverse-transcribed into complementary DNA and analysed by polymerase chain reaction (PCR). Use of primers designed to examine the entire coding region of TRAP gave PCR products of normal length in all three

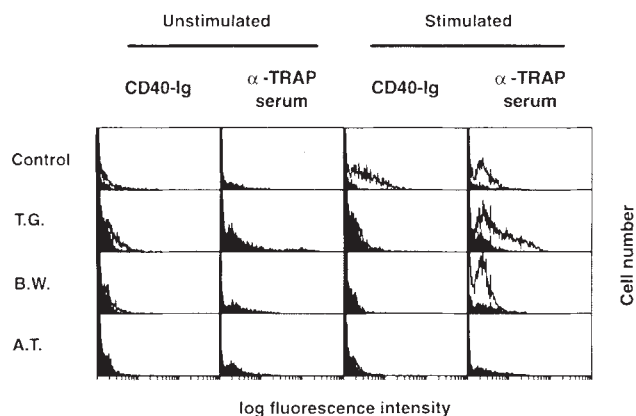


FIG. 1 Expression of TRAP on the surface of T cells in patients with HIGM1. Flow cytometry profiles of unstimulated and stimulated PBL from patients T.G., B.W. and A.T. and a healthy control after staining with CD40-Ig and anti-TRAP serum.

METHODS. PBL were either left unstimulated or were activated by PMA (20 ng ml^{-1}) and Ca^{2+} -ionophore A23187 (125 ng ml^{-1}) for 5 h, the optimal time point for TRAP surface expression with this stimulus (our unpublished results). Resting and activated PBL were stained with the biotinylated chimaeric molecule CD40-Ig, generated by fusing the extracellular domain of CD40 to the heavy chain of IgG1¹⁸. After washing, cells were reacted with extravidin-FITC (Sigma) and analysed on a Profile flow cytometer (Coulter Electronics). To control for specificity of the signal, PBL were stained in parallel with biotinylated IL4R-Ig, in which the extracellular domain of the IL-4 receptor was fused to the heavy chain of IgG1 (ref. 18; control curves filled in black in the CD40-Ig flow cytometry profiles). Unstimulated and activated PBL were also stained with TRAP-specific rabbit antiserum, washed, and reacted with a FITC-conjugated goat anti-rabbit F(ab')₂ preparation (Sigma). Specificity of staining was controlled by parallel staining with preimmune rabbit serum (control curves filled in black in the anti-TRAP serum profiles). TRAP-specific antiserum was generated by cloning a fragment of TRAP cDNA⁶ encoding amino acids 38-183 into the His-tag vector pQE-31 (Diagen, Hilden, Germany). The polypeptide was expressed as a fusion protein in *Escherichia coli*, purified, and used in a denatured state for immunization of rabbits.

patients (data not shown). The PCR-amplified cDNA of the TRAP coding region was then cloned into suitable vectors and fully sequenced in each case. Sequence analysis of cDNA obtained from T.G. revealed a point mutation at nucleotide 475, turning the respective codon into a stop codon (Fig. 2). The cDNA obtained from B.W. had another point mutation in the same codon, resulting in amino-acid exchange (tryptophan to glycine). The only base change detected in the cDNA of A.T. was a point mutation at nucleotide 163 resulting in an exchange of the neutral methionine for the positively charged arginine in the transmembrane domain of TRAP (Fig. 2). Apart from the described point mutations, the cDNA sequences were identical to the previously determined sequence⁶ except for the silent exchange from cytosine to thymidine at position 204 in all three patients.

The cDNA sequencing results, when combined with the flow cytometry data, can be interpreted as follows. In T.G., activated T cells synthesize a truncated TRAP molecule which is expressed on the cell surface (as shown by reactivity with anti-TRAP serum) but cannot bind CD40. In B.W. the drastic exchange from tryptophan to glycine apparently disrupts the conformation of the TRAP molecule expressed on the cell surface so that interaction with CD40 is not possible. In A.T. the introduction of a positive charge into the transmembrane domain abrogates cell-surface expression of TRAP. This agrees with previous findings which demonstrate that a positive charge in a transmembrane domain constitutes a signal for endoplasmic degradation¹⁵.

To exclude a malfunction of B cells in HIGM1 definitively, we tested their capacity for immunoglobulin synthesis. First, PBL from T.G., B.W. and A.T., as well as PBL from age-matched controls, were cultured in the presence of pokeweed mitogen,

TABLE 1 Secretion of immunoglobulin isotypes by cultured PBL from individuals with HIGM1

Patients and controls	Stimulus	Immunoglobulin secreted (ng ml ⁻¹)			
		IgM	IgG	IgA	IgE
T.G.	PWM	<	50	<	<
B.W.	PWM	<	<	<	<
A.T.	PWM	250	<	<	<
Con O.I.	PWM	1,300	150	300	<
Con D.G.	PWM	1,500	400	1,400	<
T.G.	xCD40 + SAC	1,570	50	<	<
B.W.	xCD40 + SAC	1,200	<	<	<
A.T.	xCD40 + SAC	3,000	<	<	<
Con O.I.	xCD40 + SAC	170	100	170	<
Con D.G.	xCD40 + SAC	1,600	950	<	<
T.G.	xCD40 + SAC + IL-10	62,000	1,490	6,300	NT
B.W.	xCD40 + SAC + IL-10	23,000	300	600	NT
A.T.	xCD40 + SAC + IL-10	115,000	1,200	1,600	NT
Con O.I.	xCD40 + SAC + IL-10	10,700	1,900	1,400	NT
Con D.G.	xCD40 + SAC + IL-10	34,900	9,700	12,300	NT
T.G.	xCD40 + SAC + IL-4	<	65	<	19.7
B.W.	xCD40 + SAC + IL-4	<	70	<	34
A.T.	xCD40 + SAC + IL-4	1,580	75	<	39
Con O.I.	xCD40 + SAC + IL-4	600	100	180	7
Con D.G.	xCD40 + SAC + IL-4	75	200	300	17

PBL (2×10^5 in 200 μ l) from patients T.G., B.W. and A.T. and two controls (Con O.I., age-matched control for T.G. and B.W.; Con D.G., age-matched control for A.T.) were cultured in the presence of pokeweed mitogen (PWM). In parallel experiments 5×10^4 PBL were cocultured with 5×10^3 irradiated Ltk⁻ cells transfected with human Fc receptor (Fc γ RIICDw32) in the presence of anti-CD40 monoclonal antibody 89 (0.5 μ g ml⁻¹) and *Staphylococcus aureus* Cowan I (SAC), essentially as described elsewhere^{11,17}. Interleukin-10 (IL-10) (100 ng ml⁻¹) or interleukin-4 (IL-4) (50 U ml⁻¹) were added to some cultures in the CD40 system. In control experiments in which only IL-10 or IL-4 were added without crosslinking of CD40 in the presence of SAC, B cells died within 48–72 h without producing immunoglobulin (data not shown). Supernatants were collected from all cultures on day 10 and immunoglobulin levels determined with immunoglobulin isotype-specific ELISA assays. Before cell culture, the proportion of CD3⁺, CD4⁺, CD8⁺, CD40⁺ and CD20⁺ cells was determined in the PBL samples by flow cytometry and was found to be normal. The sign < indicates background levels; xCD40, crosslinked CD40; NT, not tested.

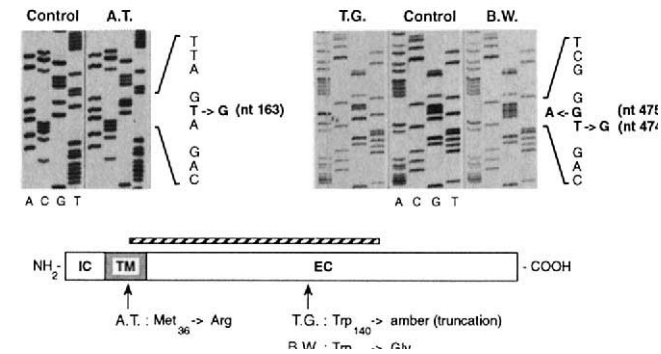


FIG. 2 Point mutations in the coding region of TRAP in patients with HIGM1. Point mutations found in TRAP cDNA of patients A.T., T.G. and B.W. are shown. Numbers indicating the nucleotide (nt) positions refer to ref. 6 (upper part of figure). The amino-acid substitutions resulting from the observed point mutations and their relative position in the TRAP molecule are also shown (lower part of figure; IC, intracellular domain; TM, transmembrane region; EC, extracellular domain). The hatched bar indicates the portion of the TRAP protein against which the rabbit antiserum was generated. METHODS. PBL from patients and controls were activated with PMA and Ca²⁺-ionophore for 4 h and total RNA was extracted. Reverse transcription of TRAP mRNA to cDNA was done according to standard methods using the primer TRAP_{low} (5'-GGCATGTGCGACTCAGAGTTTGAGTAAGCCA-3'). The generated cDNA was amplified by PCR with primers TRAP_{low} and TRAP_{up} (5'-GGCTAGAATTTCATGATCGAATACATACAACC-3'; 33 cycles, each cycle for 40 s at 94 °C, 60 s at 60 °C, and 70 s at 72 °C) and the resulting product, which encompasses the entire coding region of TRAP, cloned into Bluescript SK(+) and SK(-) (Stratagene) using the EcoRI-Sall sites incorporated in the two primers. To eliminate potential sequence artefacts caused by PCR, reverse transcription and PCR, and subsequent cloning of TRAP cDNA from activated cells of each patient, were done twice in independent experiments. For sequencing, at least 40 single clones were picked in every independent cloning experiment, pooled, and used for generation of single-stranded DNA. The entire TRAP coding region was sequenced manually.

an agent able to induce immunoglobulin synthesis by activating B cells and T cells and allowing them to cooperate. As expected, the patients' B cells could not secrete significant amounts of IgG, IgA or IgE, indicating an ineffective T-cell help for B cells (Table 1). But when the patients' B cells were triggered directly through crosslinking of CD40 in the presence of *Staphylococcus aureus* Cowan I and appropriate lymphokines, IgG, IgA and IgE were produced normally (Table 1). These data establish that B cells from HIGM1 patients are able to undergo immunoglobulin class switch *in vitro* and to produce immunoglobulin of all isotypes.

Our data demonstrate that HIGM1 is caused by a defective expression of TRAP on the surface of T cells. Our results also indicate that the mutation responsible for the defective expression of TRAP can vary in affected individuals. The mechanism leading to elevated levels of IgM and IgD in many HIGM1 patients is unclear at present. It may be a compensatory reaction of the deficient immune system to the stress of recurrent infections.

Several important conclusions regarding the function of the immune system can be drawn from the findings in HIGM1. First, the results indicate that TRAP is not required for production of IgM. In contrast, functional expression of TRAP is a prerequisite for effective immunoglobulin isotype switching and subsequent production of IgG, IgA and IgE by B cells *in vivo*. Because triggering of CD40 prevents apoptosis of germinal

centre B cells¹⁶, it is tempting to hypothesize that the virtual absence of germinal centres in spleen and lymph nodes in HIGM1 patients³ is a consequence of a disturbed interaction between TRAP and CD40. Further insights into the central mechanisms involved in T/B cell cooperation *in vivo* can also be expected from the analysis of the autosomal recessive and autosomal dominant forms of hyper-IgM³. □

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CD40 ligand mutations in X-linked immunodeficiency with hyper-IgM

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SIGNALLING for the B-cell immunoglobulin isotype switch requires T-cell-derived cytokines and T–B cell interaction, which operates primarily through the CD40 molecule on B cells with its ligand (CD40L) on activated T cells (reviewed in ref. 1). The CD40L is a type II membrane protein^{2–5} with homology to tumour necrosis factor- α and - β ^{6,7}, and has important functions in B-cell activation and differentiation^{2,4,8}. Human CD40L maps on Xq26.3–27.1 (ref. 3), the region where a primary immunodeficiency characterized by an immunoglobulin isotype switch defect (the hyper-IgM immunodeficiency syndrome, HIGM1) has been localized^{9,10}. The hypothesis that HIGM1 involves an abnormality of the

CD40L has been tested. We report here the lack of CD40L expression in four unrelated male children with the hyper-IgM syndrome. CD40L transcripts in these patients showed either deletions or point mutations clustered within a limited region of the CD40L extracellular domain. These genetic alterations with abnormal CD40L expression provide a molecular basis for immunoglobulin isotype switch defects observed in this immunodeficiency.

X-linked HIGM1 (reviewed in ref. 11) is characterized by decreased serum levels of IgG, IgA and IgE with normal or elevated levels of IgM and IgD, implying a defect in the control of immunoglobulin isotype switch^{12,13}. The role of CD40L in this process, and its co-localization with HIGM1, make the CD40L-encoding gene a strong candidate for the HIGM1 locus. A CD40 fusion protein (hCD40-H μ)¹⁴ specifically binds the CD40L expressed on activated T cells. Most stimulated T cells from control individuals bound hCD40-H μ (Fig. 1a), whereas unstimulated cells showed no staining above background (data not shown). In contrast, stimulated T cells from four unrelated male children with the HIGM1 syndrome failed to demonstrate any detectable binding of hCD40-H μ (Fig. 1a, and data not shown), whereas CD40L was found on T lymphocytes from the mothers of these patients (Fig. 1a, and data not shown). CD40L transcripts were found in activated cells from the HIGM1 patients, their mothers and in normal controls (Fig. 1b).

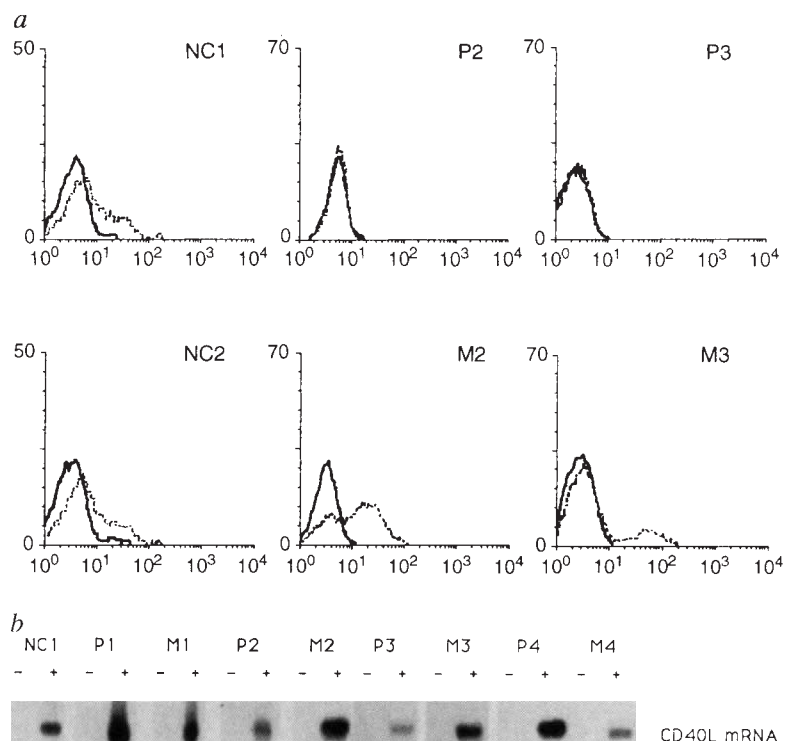


FIG. 1 Analysis of CD40L expression in HIGM1. *a*, Lack of binding of hCD40-H μ to activated HIGM1 T cells. Patients 1 (O.F.), 2 (A.Z.) and 3 (P.F.) have been previously described¹³. Patient 4 had a family history of X-linked immunodeficiency, and presented with clinical features of HIGM1 at age 3. Histograms of CD3⁺ cells from normal individuals (NC1, NC2), HIGM1 patients (P2, P3) and their mothers (M2, M3) stained with (broken lines) or without (solid lines) hCD40-H μ . Histograms depict relative cell number as a function of fluorescence intensity. *b*, Expression of CD40L transcripts in HIGM1 patients. Total RNA from resting (–) or activated (+) lymphocytes was analysed using a CD40L complementary DNA probe.

METHODS. Staining for CD40L on phorbol ester (10 ng ml^{–1}) and ionomycin (1 μ M) stimulated cells was done as described¹⁴, with an additional step using anti-CD3:PE. CD40L expression was determined by analysis of green fluorescence on electronically gated CD3⁺ cells. RNA was extracted as described²⁰ and subjected to northern blotting using standard techniques.

Moreover, the size and amount of CD40L messenger RNA appeared normal in the HIGM1 patients.

CD40L transcripts were amplified using polymerase chain reaction (PCR) for direct sequence analysis. All four patients demonstrated coding sequence abnormalities of the CD40L extracellular domain (Fig. 2a). Analysis of patient 1 transcripts revealed an in-frame deletion of 63 base pairs (bp) (bases 385–447), removing the 21 amino-acid residues from positions 116 to 136 of the CD40L. Patients 2 and 4 (P2 and P4) transcripts each showed small deletions (of 8 and 10 bp, respectively) in precisely the same region of the CD40L molecule (bases 447–457). We hypothesize that these patients may possess splice site mutations, as the deletions in these cases are cryptic donor sites (GTTACAGT and GTGTACAGT). The predicted mutant proteins would be truncated (138 amino acids in P2, 144 amino acids in P4) owing to a frameshift, with the introduction of premature stop codons (Fig. 2a). Patient 3 transcripts demonstrated a point mutation (C to A) at a CpG dinucleotide at base 407, resulting in an amino-acid substitution (Ala 123 to Glu) with a change in the hydrophobicity profile. Sequence analysis of CD40L transcripts from the mother of P3 (M3), revealed heterozygosity at base 407, thus confirming M3 as the carrier of the mutant allele (data not shown). M3 was also informative for a polymorphic microsatellite repeat present in the CD40L 3' UT region (Fig. 2b), thus allowing for the identification of the normal and affected CD40L alleles. Segregation analysis of this CA repeat in sorted CD40L⁺ and CD40L[−] cell populations from M3 revealed the normal allele in the CD40L⁺ population, whereas the CD40L[−] population carried the mutant allele (Fig. 2b). This result strongly suggests that the Ala 123 → Glu substitution results in a CD40L molecule that is unable to bind CD40.

The CD40L deletions observed in patients 1, 2 and 4 were also analysed to determine whether these deletions were new

mutational events or were maternally inherited (Fig. 2c). Amplification of the region containing these deletions revealed that the mutations in P1 and P4 seem to be inherited, as abnormal transcripts are detected in M1 and M4. In contrast, only the normal-sized product can be detected in M2, suggesting that the deletion in P2 has occurred *de novo*.

The CD40L belongs to a ligand gene superfamily (including tumour necrosis factor (TNF)- α and - β) thought to form trimeric homomultimers necessary for receptor function^{15,16}. The three-dimensional structure of the CD40L has been modelled on the known crystal structure of TNF- α ¹⁷ (C. Jongeneel, personal communication), and is predicted to form a very similar overall conformation. Some CD40L structure/function relationships may be understood in light of these HIGM1 mutations (Fig. 3). The mutation of Ala 123 to Glu in P2 concerns a residue conserved in TNF- β and mCD40L (ref. 2) thought to play a role in receptor binding¹⁷. This substitution may not affect overall expression of CD40L, despite its deleterious effects on CD40 binding. The deletion in P1 may lead to a monomeric form of CD40L, as it removes residues involved both in trimer formation and in receptor binding. The truncated CD40L forms (in P2 and P4), if expressed, delete large portions of the extracellular domain of CD40L and result in nonfunctional, and probably monomeric, forms.

Abnormal expression of CD40L provides a mechanism for the defective immunoglobulin isotype switch characteristic of HIGM1. Attempts to promote class switch in HIGM1 B cells with cytokines or activated T-cell supernatants were unsuccessful^{13,18}, and led to the hypothesis that an intrinsic B-cell defect was present. A primary T-cell defect in HIGM1 was suspected because of the recurrence of opportunistic infections (*Pneumocystis carinii*, cryptosporidium, toxoplasmosis) generally associated with anomalies of T-cell function (reviewed

FIG. 2 Characterization of CD40L mutations in HIGM1 and inheritance of mutant CD40L alleles. *a*, Direct sequence of PCR-amplified CD40L cDNA from HIGM1 patients demonstrating deletions (P1, P2 and P4) or a C to A point mutation at codon 123 (P3). For clarity, the 18 bp between the CTG triplet and the premature stop codon (boxed) in P4 has been depicted by asterisks. *b*, Segregation of CD40L microsatellite repeat in P3 and M3. The CD40L CA repeat (J.P.D. *et al.*, manuscript submitted) was PCR-amplified from stimulated lymphocyte cDNA derived from P3, unsorted M3, and CD40L⁺ and CD40L⁻ M3 populations. *c*, PCR products following amplification of the region encompassing the CD40L mutations in HIGM1. Products from control (+), patient (P) or maternal (M) PCR reactions were analysed using a 5% Nu-Sieve agarose gel. Molecular mass markers are shown on the left.

METHODS. Total RNA from stimulated lymphocytes (see legend to Fig. 1) was subjected to reverse transcriptase-PCR as described²⁰. CD40L cDNA was amplified by nested-PCR and was directly sequenced²¹ (primer sequences available from authors). Mutations were verified on both DNA strands. For CA repeat, activated cells were stained for CD40L and sorted on a FACStar flow cytometer before RNA extraction and RT-PCR.

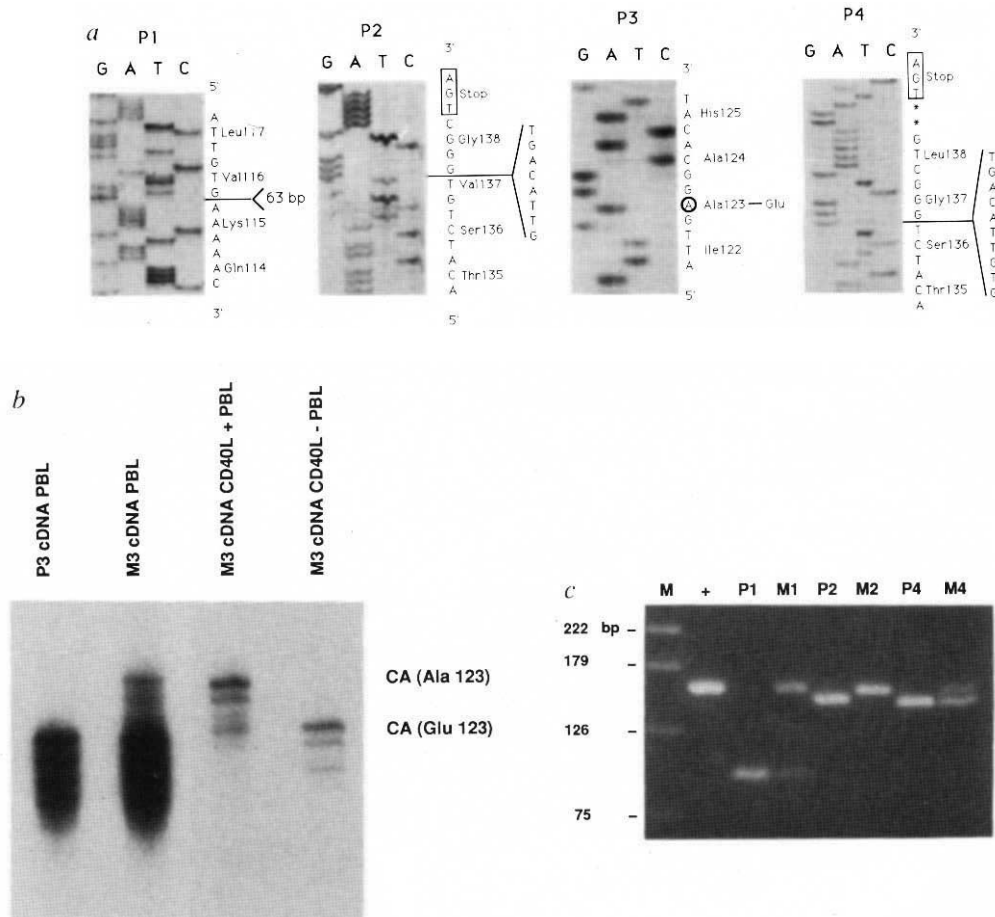
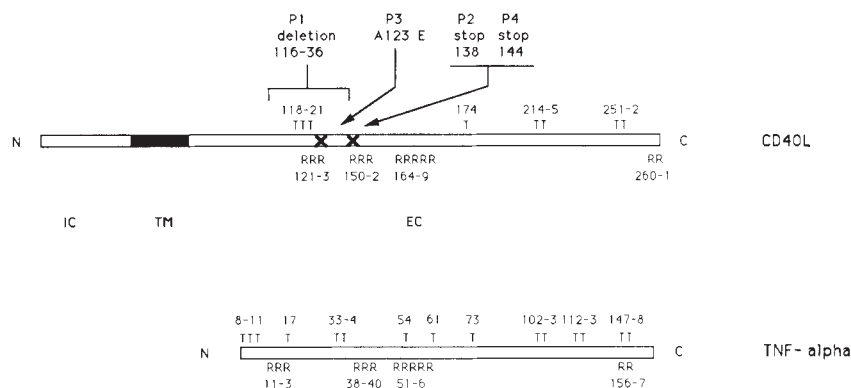


FIG. 3 Location of the CD40L mutations. CD40L compared with TNF- α with respect to sites involved in trimer formation (T) and receptor binding (R). CD40L is a type II membrane protein with extracellular (EC) C terminus and intracellular (IC) N terminus. Numbering refers to amino acids in the mature protein. Data for TNF- α taken from ref. 17. Mutations detected in the four HIGM1 patients are indicated.



in ref. 11). Moreover, isotype switch could be obtained in HIGM1 B cells after co-cultivation with Sézary's syndrome T cells¹², arguing against a primary B-cell defect. This is also supported by X-inactivation studies in obligatory carriers at HIGM1, which demonstrate a random pattern¹⁹. Our data correlate abnormalities of the CD40L with HIGM1 and result in the inability to bind CD40, a critical molecule for B-cell proliferation and differentiation. As a consequence, the complete signal for immunoglobulin isotype switch is not provided. Thus, HIGM1 emphasizes the central role of the CD40L/CD40 in immunoglobulin class switch. □

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Nodal is a novel TGF- β -like gene expressed in the mouse node during gastrulation

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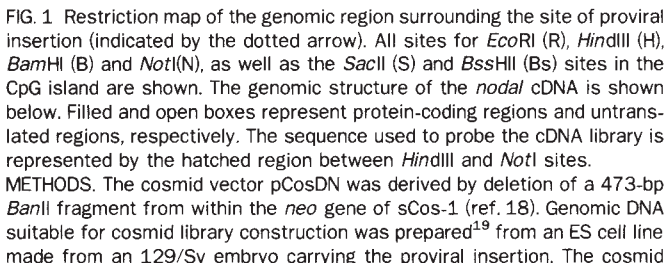
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DURING gastrulation, the three germ layers of the embryo are formed and organized along the anterior-posterior body axis. In the mouse, gastrulation involves the delamination of ectodermal cells through the primitive streak and their differentiation into mesoderm¹. These processes do not occur in embryos homozygous for a retrovirally induced recessive prenatal lethal mutation, the strain 413-d insertional mutation^{2,3}. Instead of giving rise to mesoderm, embryonic ectoderm in 413-d mutants overproliferates and then rapidly degenerates, although extraembryonic lineages remain viable². Here we isolate a candidate for the mutated gene which encodes a new member of the transforming growth factor- β (TGF- β) superfamily⁴. Expression is first detected in primitive streak-stage embryos at about the time of mesoderm formation. It then becomes highly localized in the node at the anterior of the primitive streak. This region is analogous to chick Hensen's node and *Xenopus* dorsal lip (Spemann's organizer), which can induce secondary body axes when grafted into host embryos (reviewed in

refs 5 and 6). Our findings suggest that this gene, named *nodal*, encodes a signalling molecule essential for mesoderm formation and subsequent organization of axial structures in early mouse development.

Seven overlapping cosmid clones encompassing the 413-d proviral insertion site were directly selected from a library of genomic DNA made from embryonic stem (ES) cells carrying the mutation. A map of the region flanking the provirus is shown in Fig. 1. To find the gene mutated by proviral insertion, we screened a 7.5-days-post-coitum (d.p.c.) mouse embryo complementary DNA library with a cloned genomic DNA fragment flanking the provirus. This fragment contains recognition sites for *NotI*, *Bss*HII and *Sac*II, which are characteristic of CpG islands^{7,8}, often found lying close to genes. From a screen of 1×10^6 recombinant phages, we isolated a single 1,800-base-pair (bp)-long cDNA clone which maps to both sides of the 413-d provirus (Fig. 1). The cDNA contains a long open reading frame (ORF) starting at the 5' end. Although the ORF does not begin with a methionine codon, sequence analysis of cloned genomic DNA showed that the ORF continues to an in-frame Met codon 24 bp upstream. The DNA sequence of the immediate upstream genomic subclone and of the cDNA is shown in Fig. 2a, together with the deduced protein sequence of 354 amino acids. Examination of the protein sequence revealed extensive homology to the DVR (decapentaplegic-Vg-1-bone morphogenetic protein(BMP)-related) and activin/inhibin subgroups of the TGF- β superfamily. These secreted proteins act as signalling molecules mediating cellular interactions in many tissues during development⁹. TGF- β -like proteins are most highly conserved at their carboxy termini. This region, encompassing the mature form of the protein, is generated by proteolytic cleavage of a larger precursor. An alignment of the predicted carboxy terminal 110



library was prepared essentially as described¹⁸, using packaging extracts from Stratagene and DH10B bacteria (Gibco). The entire library, 8×10^5 colony forming units (c.f.u.), was plated onto nylon filters using ampicillin selection. To isolate clones of the proviral insertion region, we took advantage of the functional *neo* gene in the provirus²⁰. One quarter of the library (2×10^5 c.f.u.) was replica-plated in kanamycin, allowing direct selection of 7 clones containing the provirus. Restriction maps of these clones were constructed using a partial digestion mapping procedure¹⁸. Fragments were subcloned into pBluescript SK (Stratagene) using standard procedures. The restriction map was confirmed by hybridization of several unique sequence flanking probes to Southern blots of 129/Sv genomic DNA (data not shown).

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amino acids of this new gene, which we have named *nodal* (see later), with those of several TGF- β superfamily members is shown in Fig. 2b. Shared structural features include a cluster of basic amino acids at the start of the conserved region, believed to be important for proteolytic cleavage, and seven highly conserved cysteine residues. Overall, *nodal* protein is from 34–39% identical in the conserved region to other superfamily members, and about 25% identical to TGF- β itself. The deduced evolutionary relationships among superfamily members are shown in Fig. 2c. The first 20 to 30 amino acids of *nodal* have the characteristics of a signal sequence, suggesting that *nodal* encodes a secreted protein.

To determine the *in vivo* expression pattern of *nodal*, both reverse-transcriptase PCR (RT-PCR)¹⁰ and whole-mount *in situ* hybridization were carried out on wild-type embryos at about the time of gastrulation. In the mouse, gastrulation starts at ~6.5 d.p.c. with the formation of the primitive streak near the junction of the embryonic and extraembryonic ectoderm at the posterior of the embryo. As development proceeds, the primitive streak elongates owing to growth of the egg cylinder and movement of cells from the embryonic ectoderm into the posterior of the streak. As a result, the anterior of the streak comes to lie at the distal tip of the egg cylinder, where a discrete structure known as the node becomes visible at approximately 7.5 d.p.c. Cells leaving the node give rise to definitive endoderm, notochord and paraxial mesoderm^{11–13}. Using RT-PCR, *nodal* RNA is detectable in total RNA prepared from pre-streak and very early streak embryos, although the amount of amplified product is far below that found for later stage embryos (Fig. 3). The first detectable expression of *nodal* by whole-mount *in situ* hybridization coincides with the appearance of the node. The

hybridization is highly localized and can be seen as a ring of staining around the node in Fig. 4. This pattern persists through the neural plate/head-fold stage, but no signal is detected by 8.5 d.p.c., coinciding with the disappearance of the node as a distinct structure. It is because of this localized expression in the node that we have proposed the name *nodal* for this gene.

Intense effort is currently focused on the molecular mechanisms underlying mesoderm induction and gastrulation in vertebrates. In *Xenopus* the earliest event involves the induction of cells in the equatorial zone to form mesoderm by factors secreted by vegetal pole cells (reviewed in ref. 14). Members of the TGF- β , fibroblast growth factor and *wnt* families of polypeptide signalling molecules have been implicated as mesoderm-inducing factors¹⁵. Other extracellular factors, such as *noggin*¹⁶, may also be involved in mesoderm formation and axial development. One problem encountered in *Xenopus* when determining the relative importance of each of these factors in mesoderm formation is the difficulty in removing them from the developing embryo and assaying the consequences. Although the mouse has limitations for experimental embryology, it does have the advantage of mutational analysis. Our study of a retroviral insertional mutant has led to the identification of the gene *nodal*, which is required for early embryonic development. Several lines of evidence suggest that *nodal* encodes an essential component of the process of mesoderm formation in the mouse. Embryos homozygous for the retroviral insertion within the *nodal* locus do not form embryonic mesoderm²; the *nodal* sequence predicts a potentially secreted protein related to TGF- β ; and *nodal* expression is detected (by RT-PCR) in embryos at the time of primitive streak formation. It has also been shown that ES cells homozygous for the insertional mutation

b

Mouse <i>nodal</i>	RHLLP	DRS	QLCRRVKFQVDFN	LIGWGSWIIYPKQYNAYRCEGECNPF	VGEEFHPT
Human BMP-2	HKQRK	RLK	SS_KRHPLY_S	DV__ND__VA_PG_H_FY_H_E_F	LADHLNS
Human BMP-3	RRKQW	IEP	RN_ARRYLK__A	DI__SE__S_KSFD_YY_S_A_QF	MPKSLKPS
Mouse BMP-4	PQRSR	KKN	KN_RRHSLY_S	DV__ND__VA_PG_Q_FY_H_D_F	LADHLNS
Human BMP-5	DYNST	EQK	QA_KKHLY_S_R	DL__QD__A_EG_A_FY_D_E_SF	LNAHMNA
Human BMP-6	DYNSS	ELK	TA_RKHLY_S_Q	DL__QD__A_KG_A_NY_D_E_SF	LNAHMNA
Mouse BMP-7	ENSSS	DQR	QA_KKHLY_S_R	DL__QD__A_EG_A_YY_E_E_AF	LNSYMNA
<i>Drosophila</i> DPP	PTRRK	NHD	DT_RRHSLY_S	DV__DD__VA_LG_D_YY_H_K_F	LADHPNS
<i>Xenopus</i> VG-1	SKLFP	TAS	NI_KKRHL_Y_E_K	DV__QN_V_A_QG_M_NY_Y_E_Y	LTEILNGS
Mouse GDF-1	PRVEV	GPV	GT_RTRRLH_S_R	EV__HR_V_A_RGFL_NF_Q_T_AL	ETLRGPGGPP
Human IHBB	RGLEC	DGRNL	CRQOFFI__R	LI__ND__A_TG_YGNY_E_S__AY	LAGVPGSA
Mouse TGF- β	LDTNYCFSSTEKN	CVRQLYI	__RKDL__	K__HE_KG_H_NF_L_P__Y	IWS_LDT
Consensus		C	LYVDF	D GW DWIAP GY A YC G CFPF L N T	

Mouse <i>nodal</i>	NHAYIQSLKRYQPHRVP	STCCAPVKTPLSLMYVDN	GRVLLHHKDMIVEECGCL
Human BMP-2	___IV_T_VNSVN SKI_ KA_V_TELSAI_M_L_ENEK_V_KNYQD_V_EG__R		
Human BMP-3	___TI_SIVRAVGVPVGIPEP__V_EKMSSL_I_FF_ENKN_V_KVYPN_T_ES_A_R		
Mouse BMP-4	___IV_T_VNSVN SSI_ KA_V_TELSAI_M_L_EYDK_V_KNYQE_V_EG__R		
Human BMP-5	___IV_T_VHLMF_DHV_ KP_A_TKLNAI_V_F_DSSN_I_KKYRN_V_RS__		
Human BMP-6	___IV_T_VHLMN_EYV_ KP_A_TKLNAI_V_F_DSSN_I_KKYRN_V_RA__H		
Mouse BMP-7	___IV_T_VHFIN_DTV_ KP_A_TQLNAI_V_F_DSSN_I_KKYRN_V_RA__H		
<i>Drosophila</i> DPP	___VV_T_VNNMN_GKV_ KA_V_TQLDSVAM_LNDQST_V_KNYQE_T_VG__R		
<i>Xenopus</i> VG-1	___IL_T_VHSIE_EDI_ LP_V_TKMSPI_M_FY_NNDN_V_RHYEN_A_DE__R		
Mouse GDF-1	AL__VLRA_MHAAA_TPGAGSP__V_ERLSPI_V_FF_NSDN_V_RHYED_V_DE__R		
Human IHBB	SSF_TAVVNQYRMRLNPGTGVNS__I_TKLSTM_M_F_DEYNIVKRDVPN_I_EE__A		
Mouse TGF- β	QYSKVL_A_YNQHN_GASA SP__V_QALEPLPIV_YVG_RKPKVEQLSN__RS_K_S		
Consensus	NHA VQTLV P P PCCVPT L IS LY D NVV:K Y NM V CGCR		

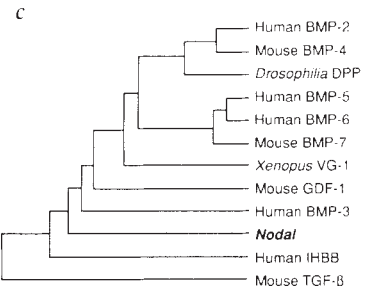


FIG. 2 Sequence analysis of *nodal*. a, Nucleotide and deduced amino-acid sequences of the cDNA and upstream genomic subclone. The nucleotide sequence begins at the *HindIII* site in the genomic subclone (the left end of the hatched region in Fig. 1). The cDNA begins at the underlined C at position 352 and the overlap of genomic and cDNA sequences continues to position 408. Exon/intron junctions, determined by sequencing appropriate genomic subclones, are marked by arrows. b, Sequence alignment of the final 110 amino acids (single-letter code) of the predicted protein sequence of the *nodal* gene with the carboxy terminal portions of several TGF- β superfamily members⁴ generated using GeneWorks (Intelligenetics). DPP, decapentaplegic; IHBB, inhibin β -chain. The 7 invariant cysteine residues are shown in bold. These and other amino acids that do not vary from the *nodal* sequence are indicated by dashes. The consensus sequence is shown below. c, Tree of alignment generated by GeneWorks, using the unweighted

pair group methods with arithmetic mean, showing the calculated evolutionary relationships of the sequences aligned in b.

METHODS. The 7.5-d.p.c. mouse embryo (CD1 strain) λ Zap cDNA library (gift from D. Weng and J. Gearhart) was plated at a density of 2×10^5 plaque-forming units per 22-cm² plate (Nunc) using 2 plates. Duplicate plaque lifts were made with GeneScreen Plus (NEN) nylon filters. Filters were hybridized with the CpG island probe (see Fig. 1) according to the manufacturer's instructions. The cDNA insert was excised from the λ Zap phage as a pBluescript SK subclone. Dideoxy sequencing was done on double-stranded DNA using ³²S-labelled dATP (NEN) and Sequenase (USB). Initial sequencing used T3 and T7 promoter primers. Subsequent sequencing primers were designed from the sequence data. This strategy was continued until the entire cDNA subclone was sequenced.

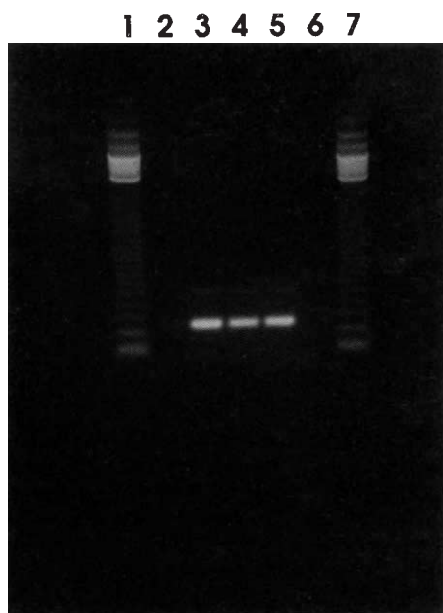
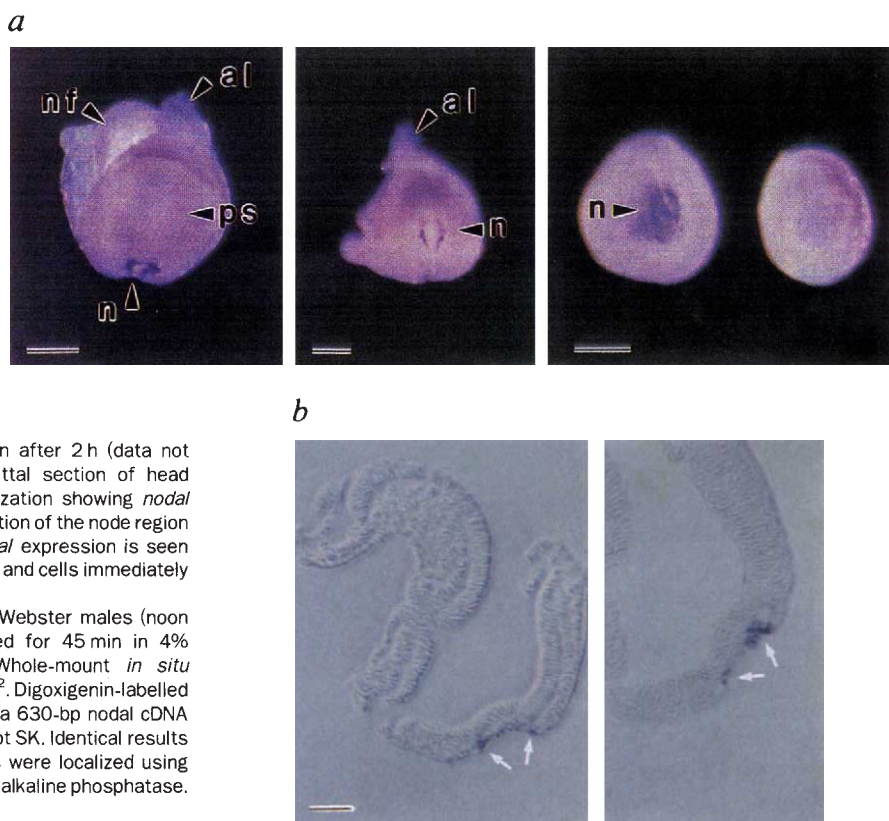


FIG. 3 Embryonic expression of *nodal* RNA. RT-PCR analysis of total RNA prepared from embryos at ~6.25 d.p.c. (pre- and very early streak stage) (lane 2); at 6.75 d.p.c. (early streak stage) (lane 3); 7.25 d.p.c. (late streak stage, but before appearance of node) (lane 4); 7.5 d.p.c. (head-fold stage with node visible) (lane 6, control (water in place of RNA); lanes 1 and 7, 123-bp ladder (Gibco). The band visible in all lanes (except control) results from amplification of a 290-bp fragment using primers lying in different exons.

METHODS. Timed pregnancies were obtained as described for Fig. 4. Reverse transcription was done on 1 μ g total RNA using specific priming. The primer used, C6, is the reverse complement of nucleotides 1,721 to 1,739 in the sequence in Fig. 1. Three rounds of fully nested PCR were then performed on the cDNA samples. The first round, using NH-2 (361 to 384) as forward primer and C6 as reverse primer, was for 20 cycles, with each cycle consisting of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min 15 s. The second round, using P1112 (1,112 to 1,131) as forward primer and P1447 (reverse complement of 1,427 to 1,447) as reverse primer, was also for 20 cycles, with each cycle consisting of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. The third round, using P1133 (1,133 to 1,153) as forward primer and RC-9 (reverse complement of 1,406 to 1,423) as reverse primer, was also for 20 cycles with each cycle consisting of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min.

FIG. 4 Localization of *nodal* RNA. *a*, Left and middle panels, whole-mount *in situ* hybridization of two head-fold-stage embryos (~7.5 d.p.c.; no visible somites), viewed from the ventral surface with posterior end up, showing expression in the node (n). The neural folds (nf) at the anterior of the embryo, and the primitive streak (ps) and allantois (al) at the posterior, show no hybridization signal. Right panel, two later primitive streak-stage embryos (~7 d.p.c.) from the same litter. The embryo on the left shows expression associated with the node. The less developed embryo at right shows a very low level of expression and no discrete node. In all cases the colouring reaction was carried out for 15 h. No specific signal was seen with sense-strand probe. Experiments performed under essentially the same conditions revealed expression of *goosecoid*²¹ RNA (from E. M. DeRobertis) in embryos before node formation after 2 h (data not shown). Scale bar, 0.2 mm. *b*, Left panel, parasagittal section of head fold-stage embryo after whole-mount *in situ* hybridization showing *nodal* expression (arrows). Right panel, high-power magnification of the node region of a more sagittal section of the same embryo. *Nodal* expression is seen in a small group of external cells (possibly endoderm) and cells immediately subjacent to them. Scale bar, 50 μ m.



METHODS. ICR female mice were mated with Swiss Webster males (noon on the day of plug is 0.5 d.p.c.) and embryos fixed for 45 min in 4% paraformaldehyde in phosphate-buffered saline. Whole-mount *in situ* hybridization was carried out essentially as described²². Digoxigenin-labelled antisense and sense strand RNA was prepared from a 630-bp *nodal* cDNA fragment (nucleotides 571-1,220) cloned in pBluescript SK. Identical results were obtained with a full-length cDNA probe. Hybrids were localized using sheep anti-digoxigenin Fab fragments, conjugated with alkaline phosphatase.

differentiate normally into mesoderm in injection chimaeras made with wild-type embryos³. All these findings are consistent with the normal *nodal* gene product encoding a secreted signalling molecule that mediates mesoderm formation. A key observation reported here is that *nodal* gene expression becomes highly localized in the node of the mouse embryo. Evidence suggests that this region plays a crucial role in the organization of mesodermal lineages along the anterior-posterior body axis. It may contain a resident population of stem cells influenced by locally produced extracellular morphogens^{13,17}. Future studies on the molecular mechanisms of *nodal* function are therefore

likely to improve our understanding of both mesoderm induction and specification as well as axis formation in the mammalian embryo. □

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Secreted *noggin* protein mimics the Spemann organizer in dorsalizing *Xenopus* mesoderm

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A DORSALIZING signal acts during gastrulation to change the specification of lateral mesodermal tissues from ventral (blood, mesenchyme) to more dorsal fates (muscle, heart, pronephros)^{1–3}. This signal, from Spemann's organizer, cannot be mimicked by the mesoderm inducers activin and fibroblast growth factor². The gene *noggin* is expressed in the organizer⁴, and could be the dorsalizing signal. Here we show that soluble *noggin* protein added to ventral marginal zones during gastrulation induces muscle, but that activin does not. Dorsal pattern can be partially rescued in ventralized embryos by injection of a plasmid that expresses *noggin* during gastrulation. The results suggest that the *noggin* product may be the dorsalizing signal from the organizer.

Previously we examined the effects of the expression of *noggin* protein (noggin) from injected messenger RNA and concluded that *noggin* can act early through a vegetal dorsalizing centre to induce a Spemann organizer⁴. To study late inductive effects mediated by organizer-derived *noggin*, it was necessary to control the time of exposure of embryonic tissues to *noggin*. To prepare active, soluble *noggin* protein, *Xenopus* oocytes were injected with *noggin* mRNA, and secreted products collected. After incubation with ³⁵S-methionine, fluorographs of SDS-polyacrylamide gels (Fig. 1) show a major secreted protein product only after injection of *noggin* mRNA. Without a reducing agent, secreted *noggin* protein had an *M_r* of ~64,000 (64K) (Fig. 1). Reduction of disulphide bonds with β -mercaptoethanol reduced the *M_r* by about half to 33K, indicating that *noggin* is secreted as a dimer (Fig. 1). Treatment of this sample with *N*-glycanase to remove *N*-linked carbohydrate chains reduced the *M_r* to ~28K (Fig. 1). A minor species of intermediate *M_r* (30K) was also present in *N*-glycanase treated samples, suggesting that there were other modifications to the peptide. These results agree closely with the reading frame of cloned *noggin* cDNA⁴, which predicts a secreted, glycosylated peptide of *M_r* ~26K, including the signal peptide.

One potential target of *noggin*, as a protein released from the organizer, is ventrally specified mesoderm in the marginal zone of *Xenopus* embryos. Explanted blastula and gastrula stage ventral marginal zones (VMZ) were treated with diluted *noggin*-conditioned oocyte medium, cultured to the late neurula stage, then assayed both for changes in morphology and for molecular markers of dorsal development. Other explants were incubated in buffer alone (see Fig. 2), with diluted medium from non-injected oocytes, or with activin. RNA isolated from the marginal zones at the end of the incubation was analysed for muscle-specific actin expression^{5,6}. Without added inducers, only dorsal marginal zones (DMZ) from the gastrula stage expressed muscle actin (Fig. 2B). *Noggin*-conditioned oocyte medium induced expression of a large amount of muscle actin RNA when added to gastrula VMZs. Neither medium from non-injected oocytes, nor purified activin had this inductive activity at the gastrula stage. In contrast, both *noggin* and activin induced muscle actin expression in VMZs when added at the blastula stage.

Ventral marginal zones treated with *noggin* became elongated, which is a characteristic of dorsal development (Fig. 2C), although the elongation was not as extensive as DMZs. Activin induced elongation only when added at the blastula stage (not shown). Explanted gastrula stage marginal zones were immunostained with an anti-notochord antibody⁷ (Fig. 2C). All DMZ explants stained for notochord, whereas VMZs incubated with medium from non-injected oocytes did not. Only one of nine activin-treated VMZs stained for notochord. Likewise, only one of nine VMZs incubated with *noggin* stained, despite their elongated appearance.

These results show that *noggin* is soluble, that it induces muscle in gastrula ventral mesoderm, but at this stage it induces notochord weakly or not at all. Thus *noggin* resembles the

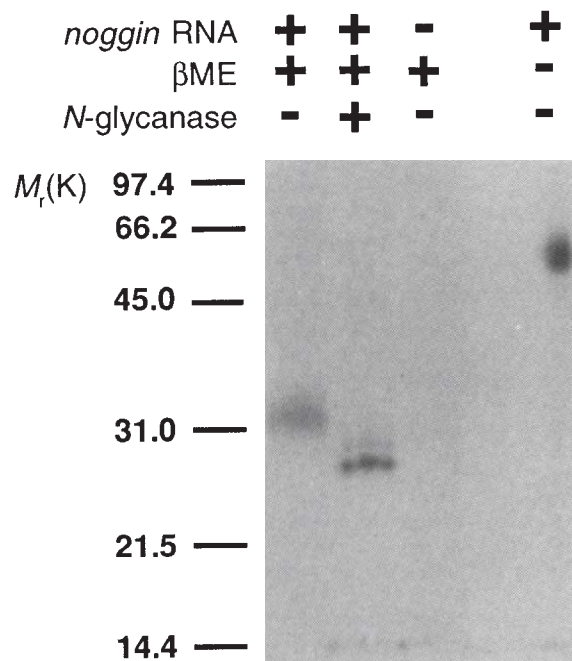


FIG. 1 Translation of *noggin* mRNA in *Xenopus* oocytes. Fluorographed SDS-polyacrylamide gel of medium from *noggin* RNA-injected *Xenopus* oocytes. Also shown is control medium from non-injected oocytes. (Samples were treated with β -mercaptoethanol (β ME) or *N*-glycanase as indicated.) METHODS. Manually dissected oocytes were injected with 50 ng *noggin* Δ 5' RNA. Oocytes were cultured for 3 d at 19 °C in 20 μ l per oocyte of Barth's solution (88 mM NaCl, 1 mM KCl, 0.82 mM MgSO₄, 2.4 mM NaHCO₃, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂ and 10 mM HEPES, pH 7.6) supplemented with 0.5 mg ml⁻¹ BSA and 0.1 mCi ml⁻¹ ³⁵S-methionine. Samples (1 μ l) of medium were analysed by fluorography after electrophoresis on 12.5% polyacrylamide-SDS gels.

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organizer signal that dorsalizes ventral tissue in the gastrula embryo².

To address how much dorsoventral pattern can be induced by noggin expression in the gastrula, a noggin-expression plasmid was injected into ventralized embryos. As a control, *Xwnt-8* was also expressed from DNA. Although both noggin and *Xwnt-8* induce dorsal development when injected as RNA into early embryos^{4,8,9}, the response of embryonic tissue changes, and *Xwnt-8* expressed from DNA no longer dorsalizes gastrula tissues¹⁰. Using a cytoskeletal actin-promoter plasmid, we directed noggin expression to the gastrula stage (Fig. 3a; *Xwnt-8* was expressed similarly). Expression of noggin from the injected DNA restored partial dorsoventral pattern to embryos (Fig. 3b). Although expression of injected DNA is variable and mosaic¹¹, the plasmid expressed much more transcript than the endogenous gene; nevertheless, the degree of rescue was modest compared to rescue by RNA injection. The rescued dorsal structures were exclusively posterior of the mid-brain, with structures that required a complete organizer being

absent. These results reinforce the idea that noggin can induce dorsoventral pattern, but cannot replace the organizer in the gastrula.

Noggin is expressed both maternally and zygotically⁴. Previous RNA injection experiments raised the possibility that maternal noggin may act at the blastula stage to dorsalize the mesoderm, thus giving rise to the Spemann organizer⁴. Zygotic noggin expression begins at the late blastula stage in the organizer itself, and reaches much higher levels than found maternally⁴. Using noggin protein secreted from *Xenopus* oocytes (Fig. 1) (and from transfected cell lines; data not shown), and noggin expression plasmids, we have shown that noggin has dorsalizing activity on gastrula-stage embryos. This activity is very similar to that from DMZ explants in recombination with VMZ². Noggin is the only known cytokine with this activity. Although the signalling pathways of activin and fibroblast growth factor are required for early mesoderm induction^{12,13}, neither these, nor other cytokines, can mimic the action of the Spemann organizer (ref. 2, and Fig. 2). Even *Xwnt-8*, which has

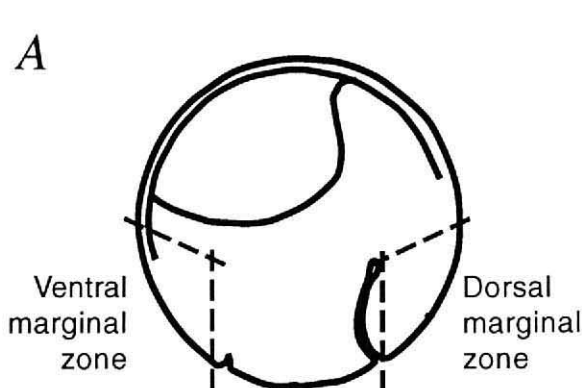
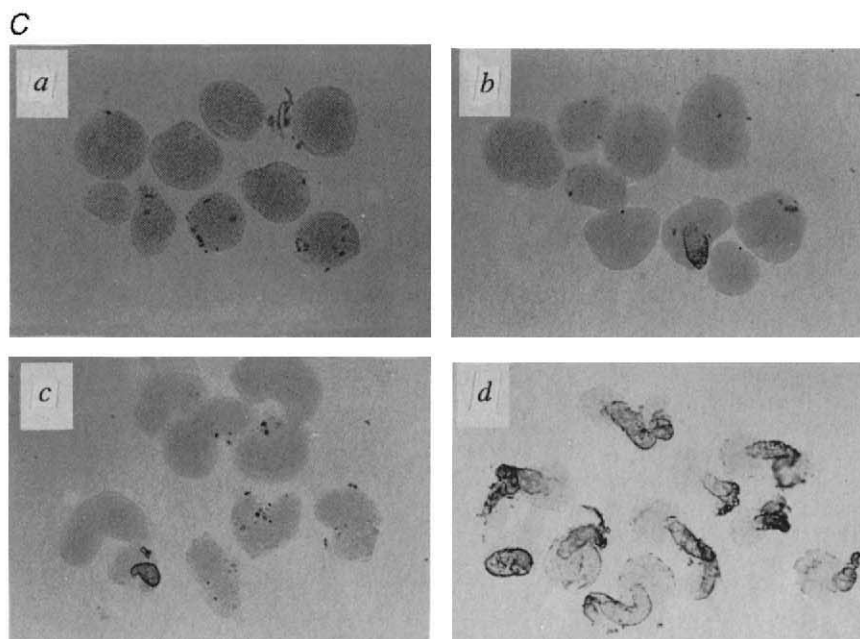
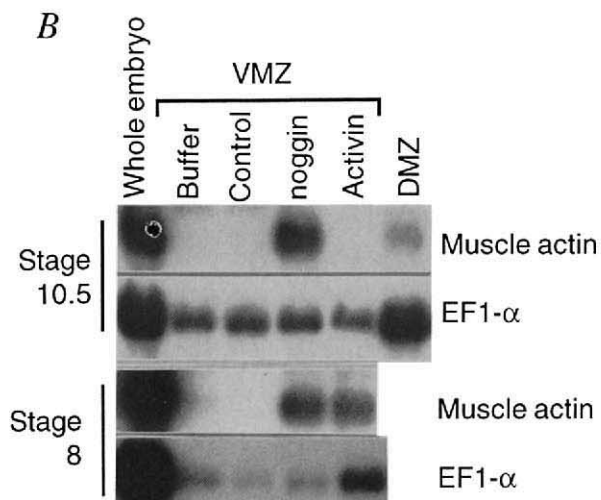


FIG. 2 Expression of muscle actin mRNA in explanted marginal zones. **A**, Gastrula-stage *Xenopus* embryo showing positions of the ventral marginal zone (VMZ) and dorsal marginal zone (DMZ) before dissection. **B**, Northern blot analysis. Ventral and dorsal MZs were dissected at either stage 8 (blastula¹⁴) or stage 10.5 (gastrula), and cultured to stage 20 (late neurula). DMZs were incubated in explant buffer only (see below). VMZs were cultured in either explant buffer alone, or explant buffer containing: a 1:4 dilution of medium from non-injected oocytes (control), a 1:4 dilution of medium from *noggin* mRNA-injected oocytes (noggin), or with 50 pM porcine activin. RNA was prepared from the explants and identically staged whole embryos at the end of the incubation and analysed by northern blotting for cardiac actin (a marker of somitic muscle), and elongation factor EF1- α (a control for RNA loading). **C**, Whole-mount immunostaining of MZ explants with an anti-notochord antibody. Specific staining is apparent as dark rod-shaped structures. All explants were cultured from stage 10.5 to 20. Treatments of the explants are as follows: **a**, VMZ with 1:4 non-injected oocyte medium; **b**, VMZ with 50 pM porcine activin; **c**, VMZ with 1:4 *noggin*-conditioned oocyte medium; **d**, DMZ with explant buffer alone.

METHODS. *Xenopus* embryos were fertilized and cultured as described¹⁵. Embryos for dissection of stage-8 MZs were first marked on the ventral side at the 4-cell stage with Nile blue¹⁶, using pigment differences between the dorsal and ventral blastomeres as a guide. Stage-10.5 VMZs constituted a ~30° segment in the marginal zone directly opposite the dorsal lip of the blastopore. DMZs were of the same approximate size but centred on the dorsal lip. Each marginal zone was cultured individually in 10 μ l explant buffer (buffer LCMR³ with 0.5 mg ml⁻¹ BSA, and 10 μ g ml⁻¹ oxytetracycline) at 19 °C until the appropriate stage for analysis.



For northern analysis, RNA was isolated from MZs as described¹⁵. Filters were hybridized first with a *Xenopus* cardiac actin probe¹⁷, and then with EF1- α ¹⁸. Other marginal zone explants from albino embryos were fixed for whole-mount immunostaining with Tor 70 antibody, which is specific for notochord⁷.

axis-inducing activity when injected early, loses the activity when the mRNA is presented later (ref. 10, and Fig. 3).

In summary, we have previously shown that maternal noggin may be part of the vegetal dorsalizing centre (Nieuwkoop centre) that induces overlying marginal zone to become dorsal mesoderm⁴. The potent dorsalizing effects of noggin protein on VMZs shown here, and the high level of noggin mRNA

expressed in the DMZ⁴, suggest that zygotic noggin may function as part of the Spemann organizer. □

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Cloning and expression of apical membrane water channel of rat kidney collecting tubule

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CONCENTRATING urine is mandatory for most mammals to prevent water loss from the body. Concentrated urine is produced in response to vasopressin by the transepithelial recovery of water from the lumen of the kidney collecting tubule through highly water-permeable membranes^{1,2}. In this nephron segment, vasopressin regulates water permeability by endo- and exocytosis of water channels from or to the apical membrane^{3,4}. CHIP28 is a water channel in red blood cells and the kidney proximal tubule⁵, but it is not expressed in the collecting tubule⁶. Here we report the cloning of the complementary DNA for WCH-CD, a water channel of the apical membrane of the kidney collecting tubule. WCH-CD is 42% identical in amino-acid sequence to CHIP28. WCH-CD transcripts are detected only in the collecting tubule of the kidney. Immunohistochemically, WCH-CD is localized to the apical region of the kidney collecting tubule cells. Expression of WCH-CD in *Xenopus* oocytes markedly increases osmotic water permeability. The functional expression and the limited localization of WCH-CD to the apical region of the kidney collecting tubule suggest that WCH-CD is the vasopressin-regulated water channel.

Rat kidney medulla messenger RNA was used in a reverse-transcribed polymerase chain reaction⁷ (RT-PCR) with two degenerate primers corresponding to the conservative amino-acid sequences in the members of the MIP (membrane intrinsic protein) family, including CHIP28 (ref. 8). The sequence of one PCR clone (mwc41) represented a potentially new water channel with 45% amino-acid sequence identity to CHIP28. To obtain a full-length clone, a rat kidney cDNA library in λ gt22 was constructed and screened using the clone mwc41 as a probe.

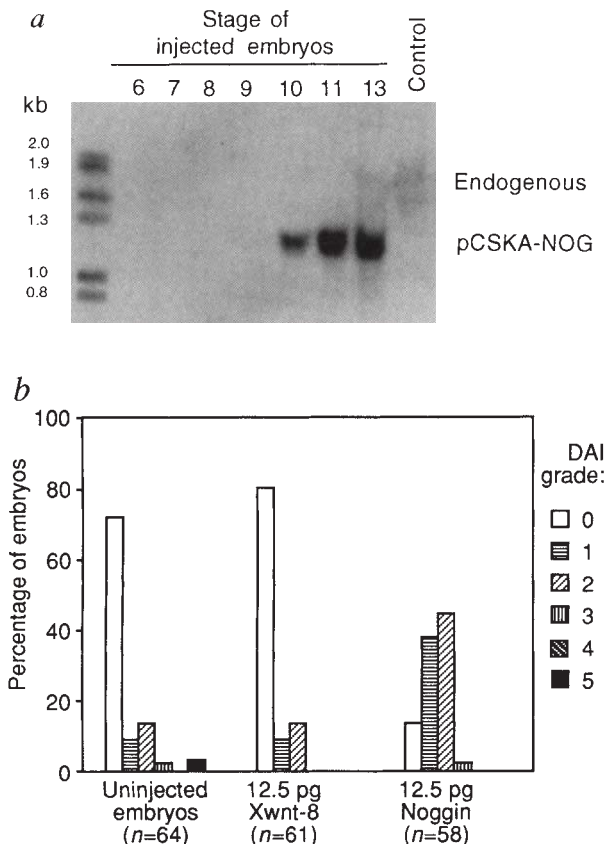
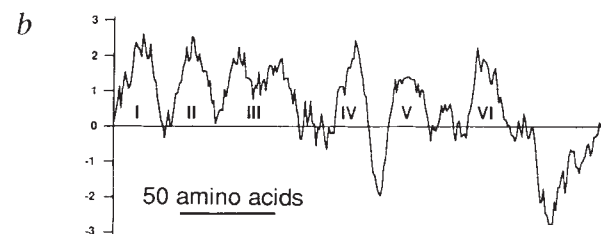


FIG. 3 Injection of noggin and Xwnt-8 expression plasmids into ventralized embryos. *a*, Northern blot of ventralized embryos injected with 50 pg noggin expression plasmid (pCSKA-NOG), showing time course of noggin expression directed by the cytoskeletal actin promoter. Injected embryos were collected at stages 6 through 9 (blastula), 10 and 11 (early gastrula), and 13 (late gastrula). Control embryos were also collected at stage 13. RNA was extracted and analysed by northern blotting for noggin, including both the abundant pCSKA-NOG transcript (1.2 kb), and the rare endogenous transcripts (1.4 and 1.8 kb). For reference, sizes of DNA standards are given in kb on the left. *b*, Distribution of embryos of varying dorsoanterior index (DAI¹⁹) grades, produced in one batch of ventralized embryos by the following treatments: no injection; injection with 12.5 pg Xwnt-8 expression plasmid (pCSKA-Xwnt-8); and injection with 12.5 pg pCSKA-NOG. Grade 0 represents the completely ventralized phenotype, and grade 5 indicates normal dorsal development. Grade-1 embryos have somites and spinal cord; grade-2 embryos also develop hindbrain; grade-3 embryos develop eye pigment. The mean DAI values are 0.6 ± 0.1 for uninjected embryos, 0.3 ± 0.1 for Xwnt-8-injected embryos, and 1.4 ± 0.1 for pCSKA-NOG-injected embryos. The number of embryos scored for each treatment is given in parentheses.

METHODS. A 794-bp *EcoRI* to *HindIII* fragment of noggin cDNA⁴ was ligated into the *Sall* and *HindIII* sites of pCSKA, which contains a ~900 bp fragment of the *Xenopus borealis* cytoskeletal actin promoter²⁰. The Xwnt-8 expression plasmid contained a 1,418 bp *EcoRI* fragment of the cDNA⁸ ligated into the *EcoRI* site of pCSKA. Varying concentrations of these plasmids were injected into embryos ventralized by ultraviolet irradiation⁸, into the marginal zone of one blastomere at the 4-cell stage. For northern blot analysis, 5 embryos were selected randomly at each stage, and RNA was extracted¹⁵. RNA filter blots were hybridized¹⁵ with a *Xenopus noggin* probe. Embryos were also grown to about stage 30 and scored for dorsal rescue according to the dorsoanterior index¹⁹.

One positive clone (WCH-CD, for water channel of collecting duct) was isolated and sequenced (Fig. 1). The longest open reading frame encodes a 271-amino-acid protein (M_r 28,928) with 42.7% sequence identity with human CHIP28 and 59.1% sequence identity with rat MIP⁹. Conserved residues in WCH-CD and the members of the MIP family¹⁰, and internal tandem repeats⁸ in the WCH-CD sequence, suggest that WCH-CD is a new member of the MIP family. Hydropathy analysis of the translated protein¹¹ suggests the presence of six transmembrane domains similar to CHIP28. In the sequence there is one putative *N*-glycosylation site and one putative phosphorylation site for cyclic AMP-dependent protein kinase.



For immunolocalization, a polyclonal antibody against the peptide corresponding to the C-terminal amino acids of WCH-CD (anti-WCH-CD/C) was raised, and fixed rat kidney slices were stained with anti-WCH-CD/C. Immunofluorescence staining was observed only in the cortical and the medullary collecting tubules and not in other nephron segments, including the proximal tubule, thin descending tubule, thick ascending limb (Fig. 3*a, d*). Specificity of the antibody staining was confirmed by the lack of staining when antiserum was preincubated with the corresponding peptide immunogen (Fig. 3*b*). Immunolocalization of WCH-CD along the nephron segments, together with RT-PCR localization of WCH-CD mRNA, is indicative of the exclusive expression of WCH-CD in the collecting tubule, unlike CHIP28, which expresses in the proximal tubule and the descending thin limb of Henle's loop⁶. A minority of the cortical collecting tubule cells stained negatively, indicating that these cells may be intercalated cells, which are known not to express water channels³. Intracellular immunolocalization examined at high magnification showed intense staining in the apical membrane of the collecting tubule cells (Fig. 3*c*). There was little staining in basolateral side of the cell. The results demonstrate that WCH-CD is localized to the apical membrane of the collect-

METHODS. Rat kidney medulla poly(A)⁺ RNA was reverse transcribed and used for 30 cycles of PCR with 5 μM each of two degenerate primers, 5'-(T/C)T(T/C/A/G)AA(T/C)CC(T/C/A/G)G(C/T/C/A/G)GT(T/C/A/G)AC-3' and 5'-AA(T/C/A/G/G/C)T(A/T/C/A/G/C/G)G(C/T/C/A/G)GC(T/C/A/G)GG(A/G)TT-3', synthesized on the basis of conserved amino-acid sequences (Leu-Asn-Pro-Ala-Val-Thr, Asn-Pro-Ala-Arg-Ser-Phe, respectively) of the MIP family⁸. The cycle was: 1 min of denaturation at 94 °C, 1 min annealing at 50 °C, and 3 min extension at 72 °C, followed by a final extension of 7 min. A band corresponding to a ~370-bp PCR product was isolated by gel electrophoresis and subcloned into the plasmid vector pCR1000 (Invitrogen). Twenty-four clones were sequenced using the fluorescence DNA sequencer 373A (Applied Biosystems) with -21M13 and M13R fluorescent primers. A clone (pMWC41) was obtained with a 369-bp insert which had 58% nucleotide-sequence identity and 45% deduced amino-acid sequence identity to human CHIP28. Another clone (prCHIP28) was a 369-bp insert of 88% nucleotide-sequence identity and 95% deduced amino-acid sequence identity to human CHIP28. The clone pMWC41 was used to screen a 2 × 10⁷ recombinant rat kidney cDNA library constructed in the *NotI/SalI* site of the λgt22 vector (BRL). Under stringent conditions (hybridization in 6 × SSPE, 50% formamide at 42 °C; washing in 2 × SSC, 0.5% SDS at 42 °C), a positive clone (WCH-CD) was isolated with a ~1.4 kb insert. The cDNA insert was subcloned into the *NotI/SalI* site of the pSPORT vector (BRL) and sequenced by the Sanger dideoxynucleotide chain termination method using Sequenase (USB).

a

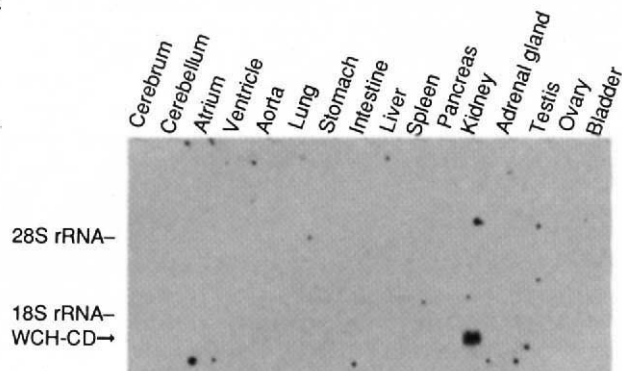
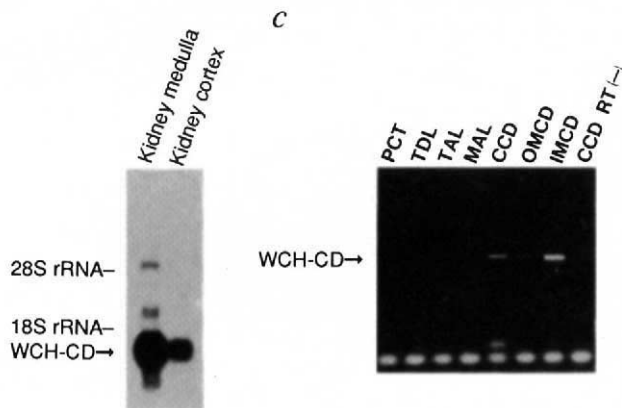


FIG. 2 Localization of WCH-CD. *a*, Northern blot analysis of WCH-CD expression in different rat tissues, showing that WCH-CD transcripts are detected only in the lane containing rat kidney mRNA. WCH-CD transcripts were not detected in lanes other than kidney mRNA by longer autoradiographic exposure up to 120 h (data not shown). *b*, Northern blot analysis of WCH-CD expression in sliced sections of rat kidney cortex and medulla. *c*, Agarose gel electrophoresis of RT-PCR products for WCH-CD mRNA on dissected nephron segments. Bands of 712 bp for WCH-CD were detected in lanes CCD, OMCD, IMCD. In Southern blot analysis, specific binding of probes to PCR bands confirmed the identity of the product (data not shown). Abbreviations: PCT, proximal convoluted tubule; TDL, thin descending limb of Henle's loop; TAL, thin ascending limb of Henle's loop; MAL, medullary thick ascending limb; CCD, cortical collecting tubule; OMCD, outer medullary collecting tubule; IMCD, inner medullary collecting tubule; RT(-), reaction without reverse transcriptase.

METHODS. For northern blot analysis, RNA extracted from several tissues was enriched for poly(A)⁺ tracts and 10 µg per lane was electrophoresed on agarose gels containing formaldehyde. Equal loading and absence of degradation was checked by staining with ethidium bromide. After transfer to nylon membranes, blots were hybridized under high stringency with the WCH-CD cDNA labelled with ³²P and autoradiographed for 24–120 h. RT-PCR of dissected nephron segments was as described^{12,13}. Briefly, 2 mm of dissected nephron segments were reverse-transcribed with random primer. Synthesized cDNA was used for 40 cycles of PCR (94 °C for 1 min, 60 °C for 1 min, 72 °C for 3 min), with specific 18-nucleotide primers for WCH-CD (5'-TGGGATCTATTTCACCGG-3', bases 522–539, and 5'-ACAGGCACTC-GGGATCAC-3', bases 1,216–1,233). PCR products were electrophoresed in 2% agarose, stained with ethidium bromide, and photographed. For Southern blot analysis, DNA was denatured and transferred to nylon membranes and hybridized with WCH-CD cDNA labelled with ³²P.



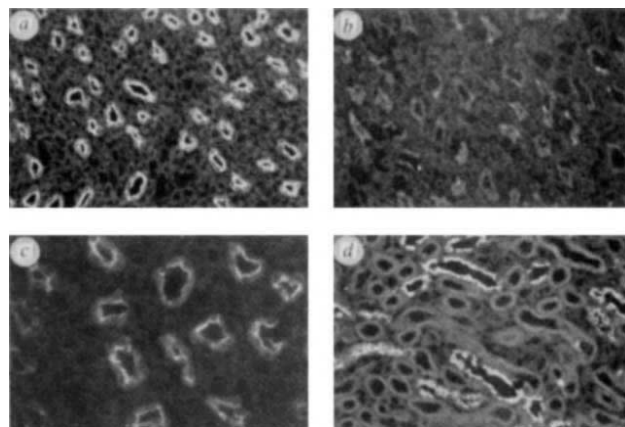
ing tubule cells. Interestingly, in addition to strong staining of the apical membrane, staining of the subapical region of the cell was seen, which, although spatial resolution is not high, may indicate localization of water channels to subapical endosomal reservoir³.

To determine the osmotic water permeability of oocytes injected with the WCH-CD transcript, the volume increase induced by an imposed osmotic gradient was measured using videomicroscopy (Fig. 4)¹⁴. The osmotic water-permeability coefficient (P_f) in WCH-CD-injected oocytes was 3.5 times greater than P_f in water-injected oocytes. Moreover, ten out of eleven oocytes injected with WCH-CD transcripts ruptured within 10 min after transfer into the hypotonic solution, whereas

none of the water-injected oocytes ruptured for more than 60 min. Osmotic water permeability in WCH-CD-injected oocytes was reduced by incubation in 0.3 mM HgCl₂, and the inhibition was reversed by β-mercaptoethanol. The activation energy (E_a) for WCH-CD-mediated osmotic water permeability was 3.6 kcal mol⁻¹, which is comparable to values reported for water channels in the red blood cell and kidney proximal and collecting tubules¹⁵. The appearance of high osmotic water permeability, the low activation energy, together with the inhibition by sulphhydryl reagents which can be reversed by reducing agents, are all characteristic of channel-mediated water permeability¹⁵ and strongly suggest that the expressed protein in the WCH-CD-injected oocytes is a water channel. Our observed P_f

FIG. 3 Immunohistochemical localization of WCH-CD in rat kidney. Sections of rat kidney medulla were incubated with rabbit antibody against the C terminus of WCH-CD (anti-WCH-CD) (*a*), or anti-WCH-CD/C serum preincubated with the corresponding peptide immunogen (*b*); magnification 40×. Staining observed in the apical domain of collecting tubule cells disappeared after preincubation with the peptide immunogen, showing the specificity of the staining. *c*, Rat kidney medulla section incubated with anti-WCH-CD/C serum (magnification 100×), showing staining of the apical membrane and the subapical region of the collecting tubule cells. *d*, Rat kidney cortex section incubated with anti-WCH-CD/C serum (magnification 40×) showing limited staining of the collecting tubule cells, and negative staining of proximal tubules.

METHODS. A peptide (VELHSPQSLPRGSKA) was synthesized corresponding to the 15 C-terminal amino acids of WCH-CD with an N-terminal tyrosine. Rabbit antisera were raised against the synthetic peptide conjugated to bovine thyroglobulin as described²⁴. New Zealand white rabbits were immunized with 0.5 ml conjugates (0.2 mg peptide) mixed into complete Freund's adjuvant. Sections (4–µm) of fixed kidney from water-restricted (2 d) rats were mounted on slides, preincubated with non-immune goat serum, and rinsed. Slides were incubated overnight at 4 °C with diluted rabbit serum (1:500). After rinsing, slides were incubated for 1 h at 25 °C with FITC-conjugated goat-anti-rabbit immunoglobulins at 1:100 dilution. Stained slides were rinsed and photographed. Immunostaining with antisera from three of five immunized rabbits produced similar results.



value in WCH-CD-injected oocytes was lower than that reported for CHIP28-injected oocytes. Possible explanations for this include the reduced translation of WCH-CD protein as a result of not using a *Xenopus* β -globin chimaeric vector⁵, or the reduced surface expression of WCH-CD in oocytes as a result of a lack of vasopressin-regulated membrane trafficking mechanisms, which are necessary to translocate water channels from subapical reservoir vesicles to the apical membrane³. Also, in

Xenopus oocytes, endosomal water-channel protein could only partially be expressed in the plasma membrane because of the nonspecific targeting of foreign protein in the egg^{16,17}.

Because vasopressin causes an enormous increase in osmotic water permeability of the apical membrane of the collecting tubule from basal level, comparable to that of other biological membranes that do not contain water channels^{15,18}, all or at least the majority of water channels in the apical membrane are considered to be vasopressin-regulated³. The presence of WCH-CD exclusively in the apical domain of the collecting tubule cells and the functional expression of water channel in oocytes, both strongly indicate that WCH-CD is indeed the vasopressin-regulated water channel of the apical and endosomal membranes of the collecting tubule. Molecular identification of the apical membrane water channel will enable the cellular mechanisms of the vasopressin-regulated water permeability of the collecting tubule cells to be directly investigated. □

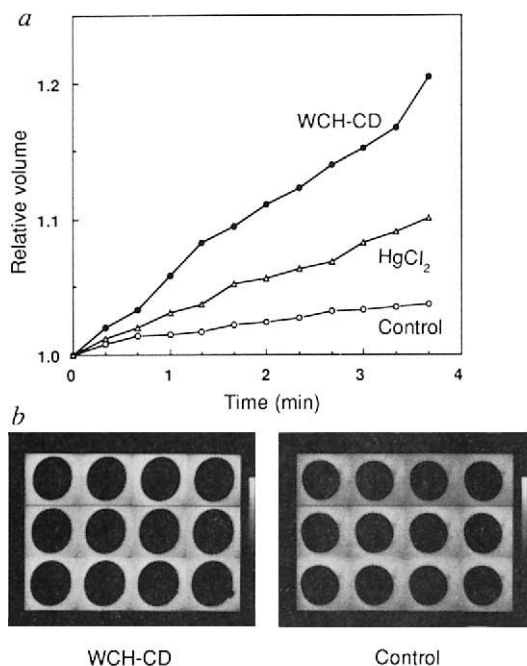


FIG. 4 Increased osmotic water permeability of WCH-CD RNA-injected *Xenopus* oocytes. *a*, Time-dependent volume increase of oocytes injected with 20 ng WCH-CD RNA (WCH-CD), or water (Control). The effects of 0.3 mM HgCl₂ were also investigated (HgCl₂). Osmotic water permeability (P_f) was 25.1 ± 1.7 (mean $\pm$ s.e.m.) $\times 10^{-4}$ cm s⁻¹ in oocytes injected with water; $83.9 \pm 18.2 \times 10^{-4}$ cm s⁻¹ in oocytes injected with WCH-CD. After 5 min incubation in 0.2 mM HgCl₂, P_f was partially inhibited to $44.5 \pm 3.6 \times 10^{-4}$ cm s⁻¹. The inhibition of HgCl₂ was reversed ($P_f = 63.1 \pm 25.8 \times 10^{-4}$ cm s⁻¹) by 15 min incubation in 5 mM β -mercaptoethanol following the incubation in 0.3 mM HgCl₂. Activation energy (E_a) for osmotic water permeability of oocytes injected with WCH-CD cRNA, estimated from the Arrhenius plot of P_f measured at 10–28 °C, was 3.6 ± 0.8 kcal mol⁻¹ ($n = 12$). *b*, Microphotographs of oocytes injected with WCH-CD RNA or water (Control). Photos were taken at 20-s intervals, in the order left to right, top to bottom. The volume of the oocytes increases with time in the case of the WCH-CD cRNA-injected oocytes.

METHODS. Capped cRNA was synthesized from WCH-CD in pSPORT vector using T7 RNA polymerase after linearization of the vector. Oocytes were prepared from female *Xenopus laevis* as described¹⁶, then injected with 20 nl water or 20 ng WCH-CD RNA ($1 \mu\text{g } \mu\text{l}^{-1}$) and incubated at 18 °C. After 24 h of incubation, oocytes were transferred from 200 mOsm (osm_{in}) to 70 mOsm (osm_{out}) of modified Barth's buffer, and osmotic volume increase was observed at 24 °C by videomicroscopy¹⁴. Oocytes were viewed by transmitted light on a Olympus phase-contrast microscope and imaged on a Hamamatsu SIT camera connected to an ARGUS-200 image-processing system. Images were stored at 20-s intervals and processed¹⁴, the area of oocyte projection being calculated by automatic summation. Relative volume (V/V_0) was calculated from the area at time 0 (A_0) and at time t (A): $V/V_0 = (A/A_0)^{3/2}$. Osmotic water permeability (P_f) was determined from the initial slope of the time course of V/V_0 ($d(V/V_0)/dt$), the initial oocyte volume ($V_0 = 9 \times 10^{-4}$ cm³), the initial oocyte surface area ($S = 0.045$ cm²), and the molar volume of water ($V_w = 18$ cm³ per mol): $P_f = [V_0 \times d(V/V_0)/dt] / [S \times V_w \times (\text{osm}_{in} - \text{osm}_{out})]$. To examine the effects of mercurial sulphhydryl reagents, oocytes were incubated in Barth's buffer containing 0.3 mM HgCl₂ for 5 min before P_f measurement. The reversal of inhibition by reducing agents was tested in a 15-min incubation in Barth's buffer containing 5 mM β -mercaptoethanol after 5 min incubation in HgCl₂.

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Uncoupling of the molecular 'fence' and paracellular 'gate' functions in epithelial tight junctions

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DURING epithelial morphogenesis, the establishment of tight junctions precedes the development of both the asymmetry in protein and lipid composition between apical and basolateral cell surfaces (the 'fence' function) and the restriction in the transport of ions and nonelectrolytes through the extracellular clefts between cells (the 'gate' function)^{1,2}. Molecular models that explain both functions envision strands of particles extending as rings in the cell's perimeter that interact with similar strands located at the

apposing cell²⁻⁵. This model accounts for the 'fence' function, because the strands prevent diffusion of protein and lipids, and also for the 'gate' function, because the interaction between strands minimizes the width of the extracellular clefts, increasing transepithelial resistance to ions and decreasing non-electrolyte permeability. Here we describe the results of energy depletion, which for the first time separates both functions: it abolishes the gate function, as determined by the dramatic decrease in transepithelial resistance, but it leaves the fence function intact, as determined by the maintenance of lipid polarity.

Depletion of ATP was achieved rapidly in confluent clone II Madin-Darby canine kidney (MDCK) cells (Fig. 1a) by using a combination of glycolytic (2-deoxyglucose) and mitochondrial (antimycin A) inhibitors. The energy status of these cells was determined by following the ATP/ADP ratio as a function of time (Fig. 1b). The decline in this ratio appeared to precede the fall in transepithelial resistance, which after 10 min decreased by 95%. Furthermore, at this point the transepithelial inulin flux more than doubled from 1.9 ± 0.3 to 5 ± 1 pmol per min-cm² after energy depletion, confirming that the gate function had been essentially abolished for non-electrolytes.

We examined tight junctions for structural correlates to this decrease in transepithelial resistance by optical and electron microscopy. Immunofluorescence of the tight-junction-associated protein ZO-1 showed no change in its distribution with respect to controls even after 30 min of energy depletion (Fig. 2). Freeze-fracture replicas of both ATP-depleted (for 10 min) and untreated MDCK cells showed no change in either the number or the geometrical complexity of tight junction strands.

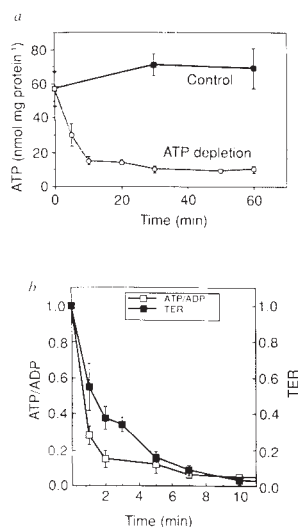


FIG. 1 Energy depletion in MDCK cells after treatment with metabolic inhibitors. *a*, Depletion of 85% of ATP was achieved within 10 min, with no further decrease for the next 50 min, as compared with uninhibited control cells. *b*, The ATP/ADP ratio decreased by 85% within the first 2 min and 95% within 10 min. This energy depletion preceded the decrease in transepithelial resistance (TER), which was also 95% complete within 10 min. The initial values for the ATP/ADP ratio and the TER were 6.5 ± 1 and 25 ± 4 ohm cm⁻², respectively. After 10 min of ATP depletion, there was no further decrease in TER during subsequent EGTA (5 mM) addition, which by itself would abolish TER^{2,5}.

METHODS. MDCK strain II were grown in DMEM medium supplemented with 10% newborn calf serum and 2 mM glutamine. Cells were passaged and plated on Anocell filter supports (Nunc) and grown in a 5% CO₂ atmosphere at 37 °C to confluency. Energy depletion was accomplished by incubating the cells for 3 h in Ringer's buffer containing 2 mM glutamine as the sole metabolic substrate (glucose-free) at 37 °C. Cells were then treated with 10 μ M antimycin A and 10 mM deoxyglucose. ATP and ADP were measured as described²⁰. TER was measured by placing the filters in a specially designed Ussing chamber and determining the transepithelial voltage resulting from the passage of a 5 μ A current pulse once a minute.

ATP-depleted and control cells differed, however, in their fracture patterns (that is, the relative distribution of particles and pits on the protoplasmic P fracture face and pits in the external E fracture face). Most strands in both ATP-depleted cells and untreated controls fractured leaving ridges on the P face and complementary furrows on the E face. Some of the strands, however, contained sections of their length with a reverse fracture pattern: the P-face ridges contained discontinuities or vacancies and the E-face furrows contained particles. We found that the percentage of the length of the strand displaying the reverse pattern increased in the cells that were ATP-depleted ($43 \pm 3\%$; $n = 5$, s.e.m.) when compared with untreated controls ($26 \pm 2\%$; $n = 5$, s.e.m.). These measurements were obtained on 25–28 μ m of total strand length in five regions from two different experiments each.

To determine whether the vacancies in the P-face ridges were due to breaks in the continuity of the strands, the P and E faces of the same tight junction strands were studied in double replicas. Examination of double replicas showed that E-face particles matched exactly the vacancies in the P-face ridges (Fig. 3). Thus, despite the reversal of the fracture face in a subpopulation of particles, a continuous ring-like structure persisted around the periphery of the cells under conditions of energy depletion, suggesting the presence of an intact fence.

To determine directly whether energy depletion affected the fence function of tight junctions, the distribution of a fluorescent lipid incorporated in the outer leaflet of the plasma membrane was assessed. Phosphatidylcholine is predominantly present in the outer leaflet of the plasma membrane in these cells^{6,7}. A fluorescent phosphatidylcholine analogue incorporated into the apical membrane of a confluent monolayer of MDCK cells remained confined to that membrane at 37 °C in the untreated control cells (Fig. 4a), as previously shown^{6,7}. An optical section (x - y plane) ~ 2 μ m below the apical surface reveals that the patches of lipid appearing below the apical surface are small round structures which are probably endocytic vesicles. An x - y section 8 μ m below the apical plane shows just minimal fluorescence due to the vesicles and no basolateral staining. Chelation of the external calcium with 5 mM EGTA (known to disrupt both gate and fence functions^{2,5-7}) during energy depletion caused a redistribution of lipid fluorescence to the basolateral surface of the cells within 30 min (Fig. 4b). The lipid diffusion

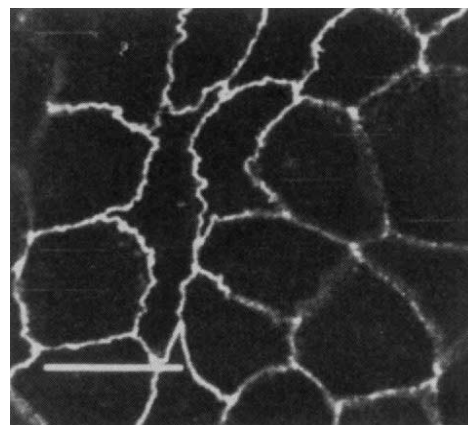
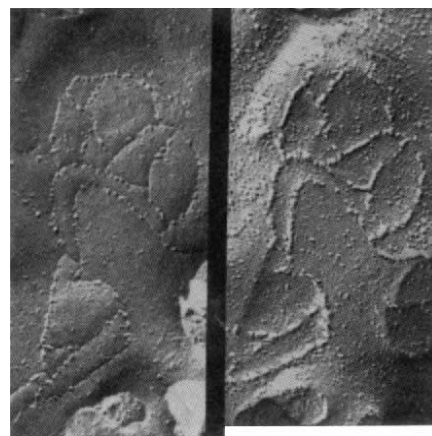


FIG. 2 Indirect immunofluorescence of ZO-1 in confluent MDCK cells subjected to 30 min of energy depletion. Tissue was fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with antibodies to ZO-1 as described²¹. The ZO-1 antibody was purchased from the Developmental Studies Hybridoma Bank (Baltimore, MD). The tissue was visualized by confocal microscopy, focused on the apical surface of the epithelium. The slight blurring of the fluorescent image was due to areas out of the apical focal plane (about 1 μ m) resulting from surface undulations of the tissue. Scale bar, 10 μ m.

FIG. 3 Double replica freeze fracture of the tight junction (TJ) region of confluent MDCK cells subjected to 10 min of energy depletion. Left panel, the E face contains the typical TJ furrows, but in this tissue they are interrupted frequently by particles. Right panel, the P face shows the TJ ridges, correspondingly interrupted by hollow regions. From this figure and from many other similar examples, it may be seen that the interruptions on the P face correspond to particles appearing on the E face. These images demonstrate that the TJ strands are continuous. The interruptions on both faces are caused by particles that have inverted their fracture patterns. Magnifications, $125,000\times$.

METHODS. Methods are described in ref. 22. Cultured cells were fixed while on the filters by immersion for 2 h at room temperature in 3% glutaraldehyde, 4% paraformaldehyde in 0.2 M sodium cacodylate buffer, pH 7.4. Cells were then infiltrated with 20% glycerol in 0.2 M sodium cacodylate buffer for 1 h at room temperature, then scraped from the filters. For double replicas, sheets of cells were deposited sandwiched between two Balzer's holders with a minimum amount of solution, and frozen by immersion in liquid propane. Fracture was by mechanical separation of the holders in the freeze-fracture apparatus. Complementary fracture surfaces were then shadowed with platinum-carbon at 45° and carbon at 90° . The resulting replicas were released by digesting the tissue with 4% sodium hypochlorite. Replicas were deposited on single-hole copper grids coated with Formvar.



through the tight junction is clearly seen in the corresponding x - y optical sections in which the fluorescent lipid delineates the basolateral membranes at 2 and 8 μm below the tight junction, bearing a similarity to Fig. 2. In contrast, energy depletion by itself does not affect the plasma membrane distribution of the fluorescent phosphatidylcholine even after 30 min (Fig. 4c), although the density of endocytic vesicles appears to diminish, as seen in the corresponding x - y optical sections.

These results show for the first time that the fence and gate functions of the tight junction can be separated in an epithelial tissue. We suggest that this functional separation is possible as a result of two different types of molecular contacts composing the tight junctional strands. The fence function depends on the contact between the particles forming the strand within each cell. This particle-to-particle contact must be extremely close, because phosphatidylcholine molecules having head groups of about 0.7 nm diameter are sterically hindered from diffusion to the basolateral membrane. Also, the fact that the tight junction of MDCK cells is composed, on average, of three strands con-

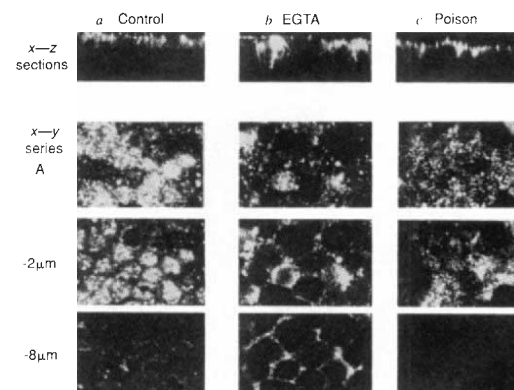
tributes to the fence function. It would similarly be expected that membrane proteins, being much larger than phosphatidylcholine, would also be constrained by the tight junctional strands to either the basolateral or apical surfaces. The tight junction fence, however, may be of secondary importance to proteins that are normally tethered to the cytoskeleton^{8,9}.

We hypothesize that the gate function (measured by transepithelial resistance and inulin flux) depends on the contact between strands located in apposing cells. The subtle but significant alteration in the freeze fracture pattern suggests that this contact is rapidly altered by energy depletion. (This finding may indicate that phosphorylation is involved in the maintenance of the gate function but not of the fence function^{10,11}.) These and other results^{2,11-15} suggest that small changes in the strand-to-strand contact can induce large changes in transepithelial resistance.

This view differs from that attempting to relate transepithelial resistance to either the number of strands or the geometrical complexity of the strands forming the tight junction^{3,4,16}. Also,

FIG. 4 Optical sections of confluent MDCK cells obtained on a confocal microscope (Bio-Rad MRC-600, or Zeiss), showing the fluorescence of lissamine-rhodamine phosphatidylcholine incorporated into the apical membrane. The top row of photographs are x - z series. Below each one of these are corresponding optical sections in the x - y plane at the apical surface ('A'), and approximately 2 and 8 μm below the apical surface (designated -2 μm and -8 μm , respectively). a, Control tissue, incubated for 30-60 min in Ringer's buffer at 37°C . Fluorescence was overwhelmingly on the apical membrane; the ratio of fluorescence obtained in the apical x - y optical section divided by that obtained 8 μm below the surface was 4.7 in this experiment. The average for 10 such measurements was 4.7 ± 1.1 . The areas of fluorescence 2 μm below the apical membrane are seen as rounded intracellular structures which are probably endocytosed membrane vesicles. b, Calcium-free condition, achieved by incubation for 30 min in EGTA containing (5 mM) Ringer's buffer at 37°C . The fluorescence pattern changed dramatically, becoming distributed from the apical to the basolateral membranes: the fluorescence ratio between the apical x - y section and the one 8 μm below the surface was 1.9 for this experiment. In 10 similar experiments, this ratio was 1.4 ± 0.2 , significantly different from control ($P < 0.01$). The x - y sections show dramatic alterations in pattern as the lipid fluorescence clearly delineates the basolateral membranes beneath the TJ. c, Energy depletion by itself for 30 min did not significantly alter the fluorescence pattern from control; for this experiment, the fluorescence ratio between the apical x - y section and that 8 μm below the surface was 3.5; the average ratio was 3.0 ± 0.3 ($n=10$), not significantly different from the control. The only difference from control may be a decrease in endocytic vesicle density, as seen in the x - y section obtained 2 μm below the surface. Scale was approximately 50 μm for the total horizontal dimension in each figure.

METHODS. Fluorescent lipid incorporation in the plasma membrane was



achieved by a modification of ref. 23. Confluent MDCK cells were first incubated for 2 h at 37°C in Ringer's buffer with glutamine (see legend to Fig. 1), followed by further incubation for 1 h at 10°C in this buffer on the basolateral side and the same buffer containing a 1:1 mixture of 25 μM defatted bovine serum albumin and the fluorescent lipid 1-palmitoyl-2-caproyl-*sn*-glycero-3-phosphocholine-*n*-(lissamine rhodamine β -sulphonyl) (Avanti Polar Lipids, Alabaster, AL) on the apical side. After washing 3 times in buffer at 10°C , filters were incubated at 37°C as described above and subsequently viewed in the confocal microscope in either the x - z or the x - y mode to obtain two-dimensional optical sections through the tissues.

the present hypothesis is more consistent with models in which the tight junctions are constructed of protein macromolecules rather than lipid micelles^{6,7}. The possibility of affecting independently the fence and gate functions of tight junctions may have important implications in a variety of tissues, such as the blood-brain barrier and the intestinal epithelium, in which the gate characteristics can be physiologically modulated¹⁷⁻¹⁹. Furthermore, this functional separation could affect the interpretation of renal ischaemia experiments in which the appearance of basolateral proteins in the apical membrane has been assumed to occur through the opened tight junctions¹². □

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Sulphation requirement for GlyCAM-1, an endothelial ligand for L-selectin

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L-SELECTIN participates in the initial attachment of leukocytes to the vascular endothelium¹⁻³. On lymphocytes, it mediates binding to high endothelial venules of lymph nodes. As a selectin⁴⁻⁶ it functions as a calcium-dependent lectin^{7,8} recognizing carbohydrate-bearing ligands on endothelial cells⁹⁻¹¹. Two lymph node ligands for L-selectin have been identified as sulphated glycoproteins of $M_r \sim 50K$ and $\sim 90K$, called Sgp50 and Sgp90 (ref. 10). The recently cloned Sgp50 (ref. 12), now designated GlyCAM-1, is a high endothelial venule-associated, mucin-like glycoprotein containing predominantly O-linked carbohydrate chains. Sialylation of GlyCAM-1 is necessary for its ligand activity^{9,10,13} and a role for fucosylation is suspected¹³. We have used chlorate as a metabolic inhibitor of sulphation, and report here that GlyCAM-1 has an additional requirement for sulphate.

Because fucose-sulphate rich polysaccharides and sulphatide¹⁴ bind to L-selectin (MEL-14 Ag, LAM-1, Leu-8) we used ³⁵S-sulphate to label endothelial ligands for L-selectin, leading to the identification of GlyCAM-1 and Sgp90 (ref. 10). To determine whether sulphation of the GlyCAM-1 carbohydrate chains was essential for activity, we used several invertebrate arylsulphatases to remove sulphate, but found that none were active. Therefore we did metabolic experiments with chlorate¹⁵, an inhibitor of ATP-sulphurylase, the first enzyme in the synthesis of 3'-phosphoadenosine 5'-phosphosulphate (PAPS), the high energy donor of sulphate. We established that chlorate substantially suppressed ³⁵S-sulphate incorporation into lymph node macromolecules (by $\sim 90\%$), while having no effect on protein synthesis (Fig. 1). Detergent lysates from ³⁵S-sulphate-labelled lymph nodes were precipitated with several reagents that recognize GlyCAM-1. In the absence of chlorate, an anti-peptide antibody¹² directed to the GlyCAM-1 polypeptide precipitated a broad band, encompassing the functional $\sim 50K$ ligand recognized by an L-selectin-immunoglobulin chimera (LEC-IgG)¹⁶. With culture in chlorate, the band precipitated by the antibody was substantially reduced (Fig. 2a) to 12% of the control level of counts (Fig. 3a). Chlorate treatment resulted in similar reductions in the ³⁵S-sulphate-labelled bands precipitated by Limax agglutinin (sialic acid specific) and Aleuria aurentia lectin (fucose-specific) (Figs 2a, 3a). In contrast, LEC-IgG failed to precipitate a detectable $\sim 50K$ band from the chlorate-treated culture (Fig. 2a); correspondingly, only a background level of counts was detected (Fig. 3a). The simplest interpretation of these findings was that chlorate exerted no

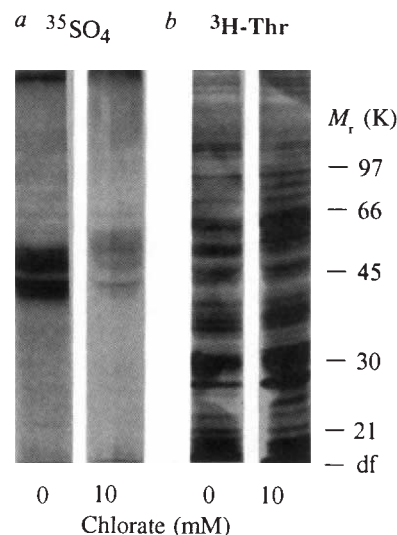


FIG. 1 Chlorate inhibits sulphate incorporation but not threonine incorporation into lymph node macromolecules. Lymph nodes were metabolically labelled in organ culture with a, ³⁵S-sulphate or b, ³H-threonine in the presence or absence of chlorate. Detergent lysates were subjected to acetone precipitation and analysed by SDS-PAGE.

METHODS. Pooled mesenteric and peripheral lymph nodes from female ICR mice (40 mg wet weight per condition) were incubated with (a) 125 μ Ci ³⁵S-sodium sulphate (ICN) or (b) 125 μ Ci of L-3-³H-threonine (Amersham) in the presence or absence of 10 mM sodium chlorate (Aldrich) in 0.5 ml of RPMI-1640 medium (1/10 sulphate concentration for sulphate labelling or threonine-free for threonine labelling) with 25 mM HEPES for 4 h at 37 °C. Tissue was extracted with 1 ml lysis buffer (2% Triton X-100 (Boehringer-Mannheim) in PBS containing 1 mM PMSF (Sigma), 1% (v/v) aprotinin (Sigma), 10 μ g ml⁻¹ pepstatin (Boehringer-Mannheim) and 0.02% Na₂S₂O₃¹⁰. Centrifuged lysates (10,000g, 30 min) were precipitated with cold acetone (80% at 4 °C). Precipitates were solubilized in Laemmli sample buffer (with 2-mercaptoethanol) and electrophoresed on a 10% SDS gel with fluorography. Molecular mass markers were phosphorylase B (97.4K), BSA (66.2K), ovalbumin (45K), carbonic anhydrase (31K), soybean trypsin inhibitor (21.5K). df, Dye front.

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effect on the overall sialylation or fucosylation of GlyCAM-1, because the reduction in both Aleuria lectin and Limax agglutinin precipitated counts closely paralleled the reduction in sulphate incorporation. But the binding of L-selectin to GlyCAM-1 was completely abrogated by culture in chlorate.

To confirm directly that the fucosylation of GlyCAM-1 was not affected by chlorate, we used ^3H -fucose as a metabolic

precursor (Figs 2*b*, 3*b*). The anti-peptide antibody precipitated equivalent bands and counts from detergent lysates of the chlorate and control cultures. But LEC-IgG precipitated no GlyCAM-1 band and only negligible counts from the chlorate-treated culture, whereas a prominent band was evident in the control.

To determine the effect of chlorate on the synthesis of the

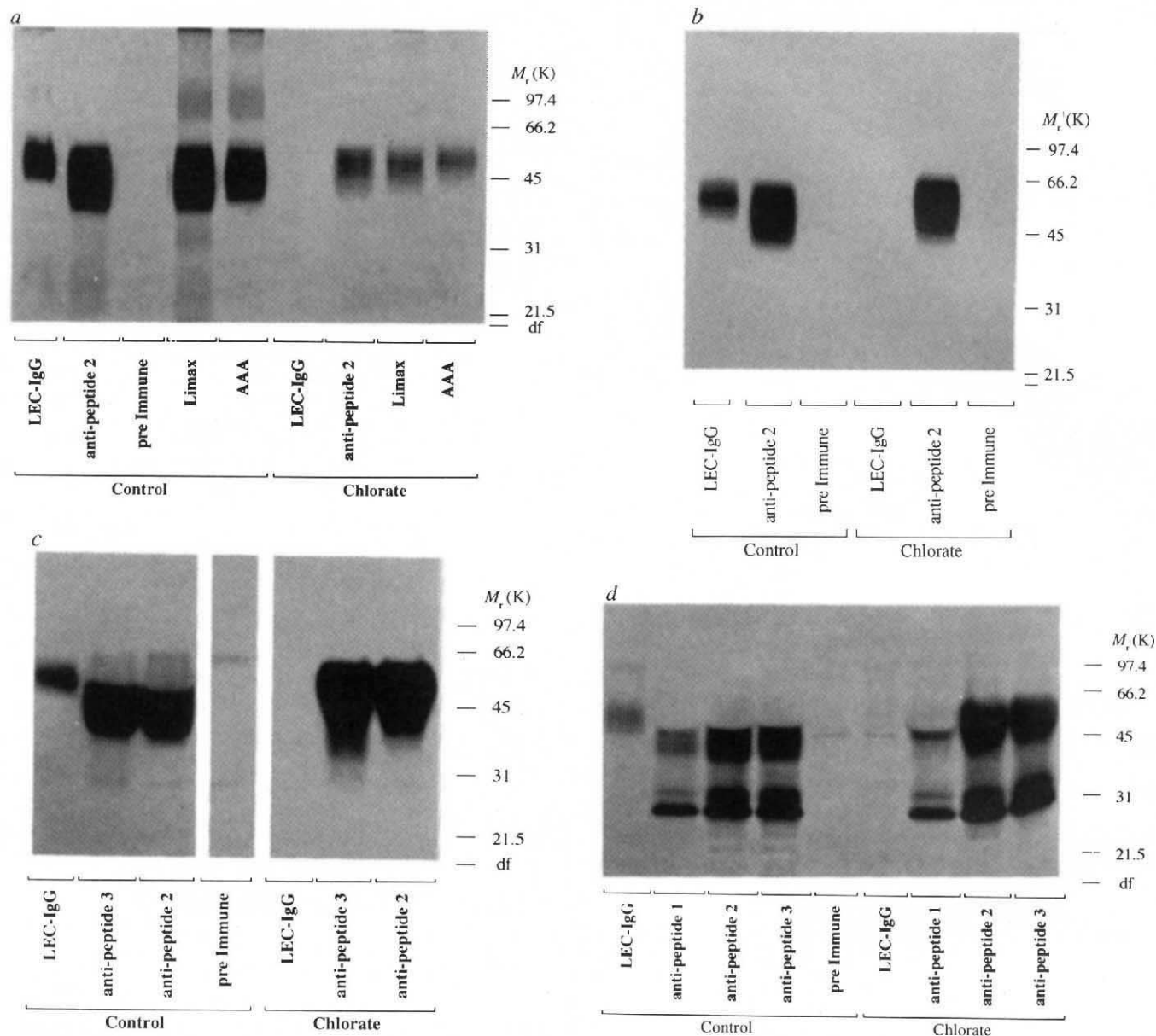


FIG. 2 Inhibition of GlyCAM-1 sulphation eliminates its interaction with L-selectin. Mouse lymph nodes were metabolically labelled in organ culture with *a*, ^{35}S -sulphate; *b*, ^3H -fucose; or *c*, *d*, ^3H -threonine in the presence or absence of chlorate. *a*, *b*, *d*, Detergent lysates or *c*, conditioned medium were precipitated with the L-selectin chimera (LEC-IgG)¹⁶, rabbit anti-Sgp50 peptide antibodies (anti-peptide 1, 2, 3)¹², preimmune serum, Limax flavus agglutinin (sialic acid specificity), or Aleuria aurantia agglutinin (AAA; fucose-specific). The ^3H -fucose- and ^3H -Thr-labelled GlyCAM-1 ran slightly larger after chlorate treatment, probably because the reduced sulphation retarded electrophoretic mobility.

METHODS. Mesenteric and peripheral lymph nodes (80 mg per condition) were incubated with *a*, 250 μCi ^{35}S -sodium sulphate (ICN); *b*, 250 μCi ^3H -L-fucose (ICN); or *c*, *d*, 750 μCi ^3H -threonine in the presence or absence of 10 mM sodium chlorate in 0.5 ml RPMI-1640 medium (1/10 sulphate concentration for *a*, *b* or 1/10 sulphate and threonine-free for *c*, *d* with 25 mM HEPES for 4 h at 37 °C). Tissue was extracted with 1.2 ml lysis buffer. The lysates were boiled for 3 min and the supernatants were

precleared with 100 μl Protein A Sepharose (Zymed) overnight. Conditioned medium collected from cultures was precleared similarly. Aliquots of the precleared supernatants were added to 10 μl of beads of LEC-IgG-Protein A Sepharose beads (30 μg LEC-IgG per 10 μl beads), rabbit anti-peptide Ab-Protein A beads (10 μl serum per 10 μl beads), rabbit preimmune serum-Protein A beads (10 μl serum per 10 μl beads), Limax flavus agglutinin-Sepharose beads (20 μg protein per 10 μl beads), or Aleuria aurantia agglutinin-Sepharose beads (10 μg protein per 10 μl beads), and incubated for 4 h at 4 °C on a rocker. The beads were washed in the lysis buffer, and aliquots of beads were saved for direct counting (Fig. 3) while the remaining beads were solubilized with (*c*, *d*) or without (*a*, *b*) 2-mercaptoethanol and analysed on a 10% SDS gel. LEC-IgG and antiserum were coated on Protein A-Sepharose beads by rocking overnight at 4 °C. Lectin beads were prepared by coupling Limax agglutinin (Calbiochem) and AAA (Boehringer-Mannheim) to CNBr-activated Sepharose 4B (Sigma). In the anti-peptide antibody lanes run with reduction (*c*, *d*), the ~50K component was compressed by the immunoglobulin heavy chain.

protein core of GlyCAM-1, we used ^3H -threonine. Because GlyCAM-1 is secreted as well as being a surface component^{12,29}, conditioned medium was analysed. LEC-IgG precipitated a strong GlyCAM-1 band in the control (Fig. 2c), which was not present when chlorate was used. With or without chlorate treatment, two anti-peptide antibodies precipitated a band that included GlyCAM-1 but was broader. Finally, when detergent lysates of cultured lymph nodes were precipitated with LEC-IgG, the GlyCAM-1 band was clearly present in the control but was completely absent from the chlorate culture (Fig. 2c). With anti-peptide antibodies against three different peptides, a broadened 50K component was seen together with several smaller components, whether or not chlorate was present.

These results indicate that chlorate substantially inhibits the sulphation of GlyCAM-1 but allows biosynthesis of the protein core, sialylation and fucosylation of its carbohydrate chains, and secretion into conditioned medium to proceed normally. The dramatic loss of GlyCAM-1 binding to L-selectin establishes the importance of sulphation in recognition. Because the O-linked carbohydrate chains of GlyCAM-1 are extensively sulphated¹⁷ and the polypeptide has only one potential tyrosine

residue for sulphate addition¹², we conclude that the critical sulphates must modify carbohydrates. A variety of biological recognition phenomena depend on sulphate modifications of oligosaccharide chains¹⁸⁻²¹. Our results now extend this diverse range of processes to a cell-cell recognition event.

Previous work has demonstrated that E-selectin²²⁻²⁵, P-selectin²⁶, and L-selectin^{13,27,28} all recognize the fucosylated and sialylated tetrasaccharide called sialyl Lewis X as well as related carbohydrates. The generation of a preferred biological ligand for each selectin may involve distinctive modifications of a common carbohydrate structure such as sialyl Lewis X or a related structure. Sulphation may constitute such a modification for GlyCAM-1. □

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A new factor related to TATA-binding protein has highly restricted expression patterns in *Drosophila*

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THE TATA-binding protein TBP is necessary for the transcription of eukaryotic genes. Multi-protein complexes formed by TBP and different TBP-associated factors are involved in the initiation of transcription by polymerases I and II, and probably III as well¹. During the formation of an active initiation complex, TBP makes specific contacts with other proteins, for example TFIIB and RNA polymerase II (refs 2-4). Here we describe the cloning and

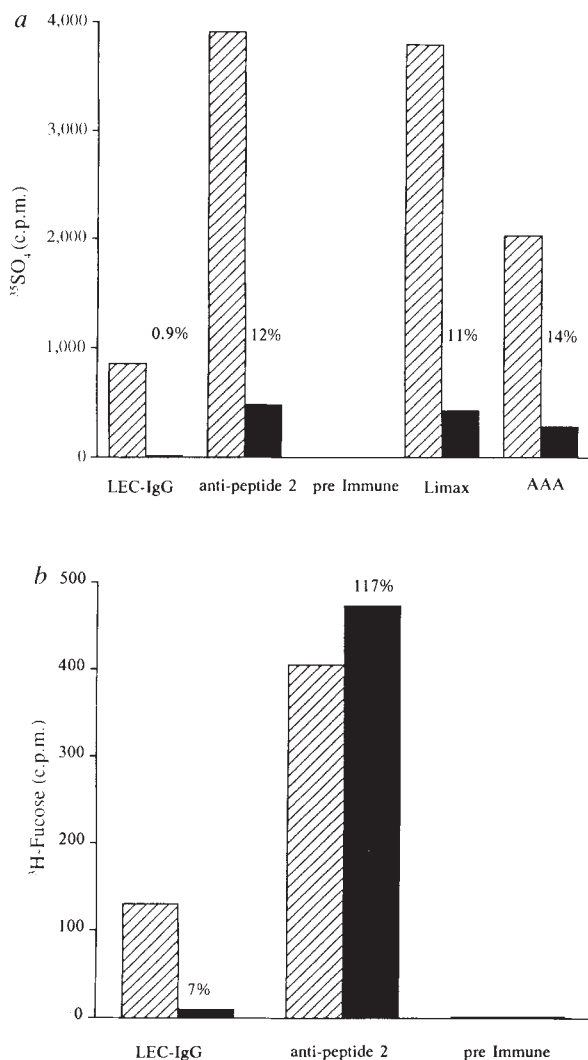
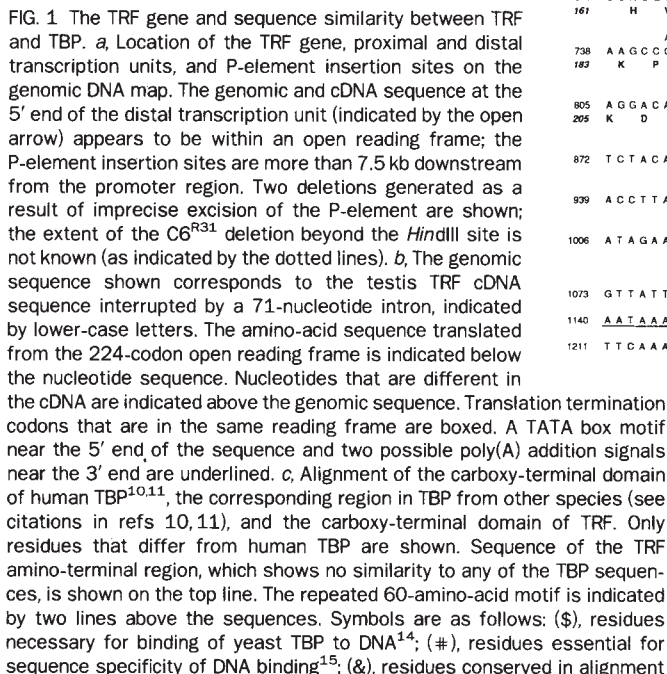


FIG. 3 Inhibition of GlyCAM-1 sulphation eliminates its interaction with L-selectin. Aliquots of the same precipitates of Fig. 2 labelled with a, ^{35}S -sulphate or b, ^3H -fucose in the presence (dark bars) or absence (cross-hatched bars) of chlorate were subjected to scintillation counting. The values shown indicate the percentage of counts obtained in the presence of chlorate as compared to the absence of chlorate.



1 TTTTTAGACATGAGAAAGTTTAGACAAATAAAATACCAAAACCGCATACAAATTTTCGTCAAGCAAT

58 A A A T A G G C A C A C A C T G G C C A C G T C G C G T G C G G A T T T G T T A T A A T T T T C G G C A A T G C A G T T T C A C T

135 T A A A G T C G C G G A C G C T G A A A G G G A C A G G G A T A A T G T G G C T G C G A C C A G C A A T G C T G C C G C C A A T C C G

212 C A C G G A C C C T G C A G C G C C A A C A G C C G T T G C C T G G T G G A G C C C A A G G A T G C A A A C A T G A G A T A C

269 G A T T G C A A T T G G T T G A C C A C T T T C A G A G G C A C T T C A T G A A T A A A T A G A A C C C A T T G A T A C A T C C

336 T A A A T T G G A A T A T C G T G G C A C C T T T C G G T G A A C T G T G A A C T G G A C C T C A A G G C A A T C A A C T

403 C G C G T A C G A G G A A C T C G G A G T A C T C G C C C A A G C G C T T T C G G G G C G T C A T C A T G C G A A T G C A C T C G C C

471 S R R T R N S E Y S P K R F T R G G I M R M H S P

544 C A G G T G C A C A G C G T A A C T T T T C G A A C G G G C A A G G T C A T C T G C A C G G T G C A C G A A C A G A T T G A G

604 R C R C T A L I F R T G G K V I C T G A R N E I E

671 G C G G C A T T G G A C T C G C G A A A G T T C G C A C G C A C T T G C A G A A G C T G G G A T T C C C C G T A A A G T C A T G G

738 A A D I G S R K F A R I C L G K L G F P P V X F M

805 A A T C A A G C T C G A A A T T T G T G C C A C C G T T G A T C T G C G T T C C C C A T C C G C C T G G A A G C C T C A A

872 E Y K L Q N I V A T T V D L R F P I R L E N L N

939 C C A G T G C A C G G C A G T T T A G T T C C T A C G A A C C G G A A G T G T T T C A G G C C T A T C T A T C C G A T G T C

1006 H V H G Q F S S Y E P E M F P G L I Y R M V

1073 A A G C G C G C A T C G T T C T C T G A T C T T C G T C A A C G G A A A G G T T G A T T T A C G G C G C C A A G T C G G C A

1140 K P R I V L L I F V N G K V Y V F T T G A K K S R

1211 A G G C A T T A T G G A C T G C C T G G A G G C G A T A C C A T A T T T T A C T A A G T T T T C G A A A G A C G T A A C T G T T

1285 K D I M D C L E A A I S P I L L S F R K T

1352 T C T A C A C A T C C A A A T G T T T T G T A T A G C T T A G T T A T A T T C C A A A C T A C A A T A T T T T A A T A T A T T T

1420 A C C T T A A C T T T T T T A G T A T A A T C T G A A A T G A A A C A A C T T A A T T A A T T T C C A T G T G T A A A T T C

1490 A T A G A A A T T G T A A G A T T T T A T T C T G G C C T T A C T A A G C A A A T T C A T T T T A T T T T A A A A A A A C T G T T

1560 G T A T T T T A A A T A A A T T T T T G T A T A T T A A T G T C T T G A A T C C A T T T C A T T C T A T T C T T T G A A T T A A T

1630 A A T A A A T T T G T A A G T C A T T T G A A T T T T A T A C A T T T T T G A C C G A T T C C T G G A A T T T G G G A A A A A T

1700 T T C A A A T C C A A T T C T G A A G T G T A C C A A C A C A C A T C C A T G A A A A G A A A A 1256

METHODS. Genomic DNA flanking the P-element was cloned by plasmid rescue⁵. Northern analysis and *in situ* hybridization^{21,22} to whole-mount embryos and adult testis revealed three transcription units. The extent and orientation of transcription for each transcription unit was determined by analysing sequences of clones from a testis cDNA library and the corresponding genomic DNA. The extent of deletions, when mobilization of the transposon resulted in imprecise excision, was determined by genomic Southern analysis.

[illegible]

characterization of a *Drosophila* gene product with considerable sequence similarity to TBP and a highly restricted expression pattern in the embryo. This TBP-related factor is a DNA-binding protein but is not likely to be a basal transcription factor. Our results suggest that TBP-related factor is a sequence-specific transcription factor that shares the DNA-binding properties of TBP.

In an attempt to identify *Drosophila* genes involved in nervous system function, we carried out transposon insertion mutagenesis⁵ and isolated two mutants that showed leg shaking under ether anaesthesia, like the *Shaker*⁶ phenotype. These mutants also showed male sterility due to immotile sperm. Both phenotypes were caused by the insertion of a P-element transposon in band 28DE on the second chromosome; revertants that arose from precise excisions of the P-element no longer exhibited either phenotype. The two P-element insertion sites were within 50 base pairs of each other, and three transcription units were found to one side of them. The P-elements were downstream from the proximal and the distal transcription units but upstream from the second transcription unit (Fig. 1a). A 1-kilobase (kb) complementary DNA, isolated from a testis cDNA library, detected a 1-kb rare transcript on a northern blot of poly(A)⁺ RNA from embryos. This transcript was detectable in primary spermatocytes in the testis of adult flies (not shown) as well as in a subset of cells in the dorsal medial region of the embryonic central nervous system (CNS) (Fig. 2). Cells present in this region of the embryonic CNS include motor neurons and glial cells^{7,8}. The expression patterns and the location of the P-elements, about 1.5 kb upstream from the transcribed sequences, indicate that this transcription unit is responsible for the mutant phenotypes, but this has not been proved as we have not been able to obtain transformants carrying stably integrated additional copies of the transcription unit. The predicted protein

product of the 1-kb transcript (Fig. 1b) showed significant similarity with the sequence of the TATA-binding protein⁹⁻¹¹ (Fig. 1c). This new gene product is therefore referred to as the TBP-related factor (TRF). Deletion of the proximal transcription unit resulted in larval lethality, but deletion of the proximal transcription unit, the unit encoding TRF, and the 3' end of the distal transcription unit caused lethality in embryos (Fig. 1a). Thus the TRF function is likely to be essential for embryonic development.

Whereas the known TBPs are at least 75% identical in the carboxyl-terminal domain, which is sufficient for TATA binding and basal transcription initiation *in vitro*^{11,12}, the 174 amino acids in the carboxyl-terminal domain of TRF are about 60% identical to TBP (Fig. 1c). The strongest sequence similarity, 80-90% amino-acid identity over a stretch of 30 amino acids (residues 166-195 in TRF), is in the segment with sequence similarity to subregions 2.3 and 2.4 of bacterial σ -factors¹⁰. A 60-amino-acid repeat found in TBP is also found in TRF. The sequence similarity of TBP to another bacterial DNA-binding protein, integration host factor¹³, is also conserved in TRF. This conserved sequence includes 7 or 8 residues at the carboxyl end of each of the two repeats. Integration host factor is known to bind in the minor groove of DNA, which is consistent with the evidence for the binding of TBP in the minor groove. Four residues in each of the two repeats are essential for yeast TBP to bind a TATA element¹⁴, and two other residues in the second repeat are important for the sequence specificity of DNA binding¹⁵. These residues were conserved in TRF as in all other known TBPs (Fig. 1c). This sequence conservation suggests that TRF can bind to the TATA sequence. To test this possibility, we expressed the 1-kb testis cDNA in bacteria, purified the TRF protein (M_r 27,000) (Fig. 3a) and assayed for its binding to the

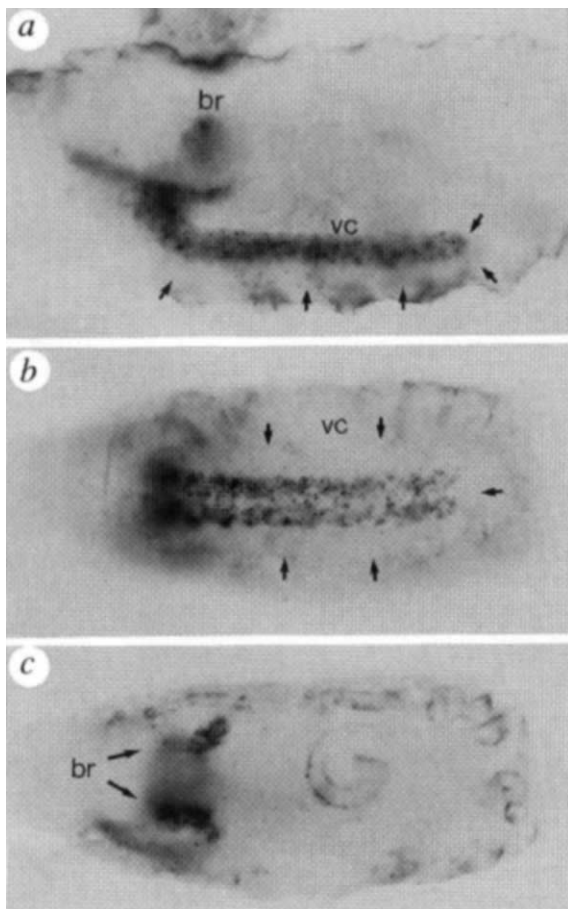


FIG. 2 Restricted expression of TRF in the *Drosophila* embryo. TRF RNA is present in a subset of cells in the medial dorsal region of the embryonic central nervous system, viewed with Nomarski optics. *a*, Lateral view of TRF-expressing cells in the brain lobe (br) and ventral cord (vc). The majority of cells in the cortex of the ventral cord and outside the nervous system show no detectable expression of TRF. *b*, Ventral view of TRF expression in the ventral cord. In *a* and *b*, arrows delineate the border of the ventral cord. *c*, Ventral view of TRF expression in part of the brain lobes. Arrows indicate signal. The light signal in the hindgut was present in negative control hybridizations.

METHODS. The 1-kb testis cDNA was labelled with digoxigenin using the Genius Kit (Boehringer Mannheim), then hybridized to fixed, stage-16 (ref. 23) wild-type embryos. The signal was detected with alkaline phosphatase-conjugated anti-digoxigenin antibody and a mixture of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrate^{21,22}.

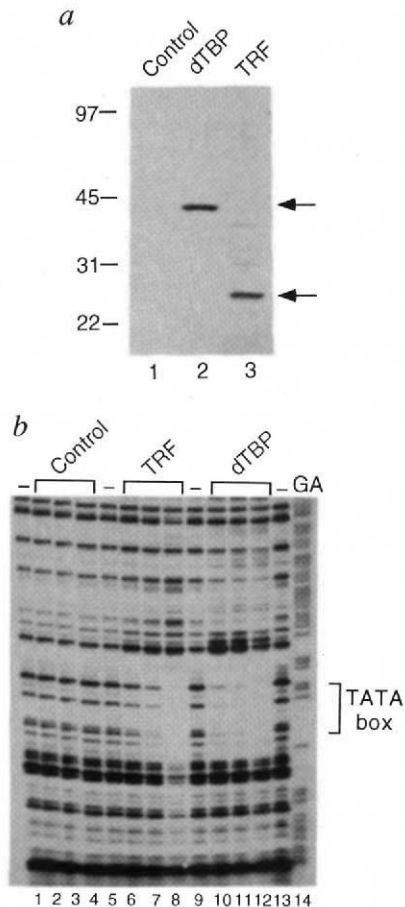


FIG. 3 TRF protein expressed in bacteria binds to the TATA element. *a*, Silver-stained protein gels showing *Drosophila* TBP (dTBP) and TRF proteins expressed in *E. coli*. Lane 1, fraction from a control extract prepared from cells containing the expression vector pET3a with no insert; lane 2, fraction containing dTBP; lane 3, fraction containing TRF. The positions of the induced proteins are indicated by arrows; migration of M_r standards ($\times 10^{-3}$) is shown on the left. *b*, TRF is a DNA-binding protein which can recognize the TATA sequence. Binding reactions for the DNase I footprint experiment were done with proteins purified from *E. coli* (shown in *a*). Lanes 1, 5, 9 and 13 are reactions without protein. Lanes 2, 3 and 4 contain increasing amounts (threefold increments) of the control fraction. Lanes 6, 7 and 8 contain increasing amounts (approximately 1, 3 and 10 ng) of recombinant TRF protein. Lanes 10, 11 and 12 are reactions with increasing amounts of dTBP. The dTBP protein produces a footprint of ~ 20 base pairs centred over the TATA box (indicated on the right). The TRF footprint extends downstream of the dTBP protected region. Lane 14 is the purine sequence of the labelled strand.

METHODS. *a*, Full-length dTBP and TRF proteins were expressed in *E. coli* using the T7 polymerase system²⁴. An *Nde*I site was created at the initiating methionine codon of TRF, and a cDNA fragment containing the entire coding region was subcloned into the T7 expression vector pET3a (ref. 25). Extracts were prepared from induced cells and fractionated on DEAE and heparin-Sepharose⁹. Proteins eluted at 0.4 M KCl were electrophoresed on a 10% polyacrylamide-SDS gel. *b*, The labelled DNA was a *Hind*III-*Hpa*I fragment from the plasmid pSG6TI (ref. 16), containing the adenovirus major late promoter TATA box. The fragment was labelled at the *Hind*III site on the non-coding strand. Binding reactions were done as described⁹, except that the reactions contained 500 ng poly(dG · dC).

TATA element in the adenovirus major late promoter^{16,17}. TRF protected the TATA sequences, as does *Drosophila* TBP⁹. TRF also protected sequences downstream from the TATA element (Fig. 3*b*).

Next we tested the ability of TRF to substitute for TBP in transcription initiation. Basal transcription dependent on the TATA element from the adenovirus E1B promoter¹⁸ was observed in the presence of *Drosophila* TBP and *Drosophila* embryo extract fractions containing TFIIB, TFIIE/TFIIF and RNA

polymerase II (Fig. 4*a*)^{9,19}. TRF, however, could not substitute for *Drosophila* TBP in this *in vitro* assay (Fig. 4*a*). The inability of TRF to support transcription initiation could result from its inability to interact with TFIIB. Whereas TFIIB associates with the complex of TBP and DNA containing the TATA element, causing a 'supershift' in the gel mobility shift assay^{4,19}, no interaction was observed when TFIIB was introduced to the complex of TRF and DNA containing the TATA element (Fig. 4*b*).

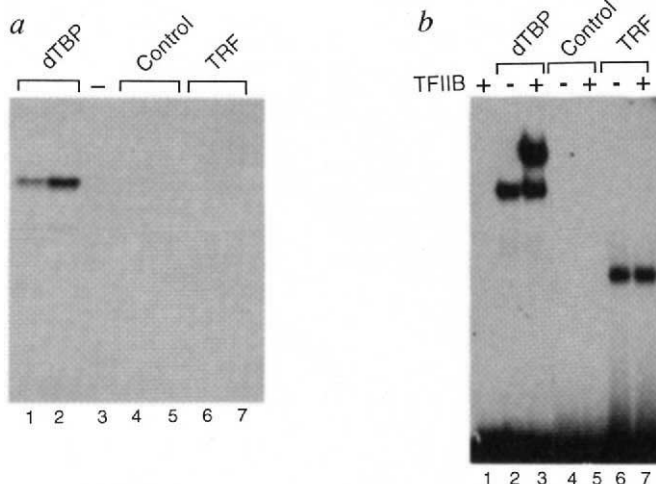


FIG. 4 TRF does not substitute for TBP as a basal transcription initiation factor. *a*, TRF has no TFIID basal transcription activity. Reconstituted *in vitro* transcription reactions were carried out with partially purified basal transcription factors fractionated from *Drosophila* embryo nuclear extracts. All reactions contained the TFIIB, TFIIE/TFIIF and RNA polymerase II fractions. Lanes 1 and 2 contain ~ 1 and 3 ng recombinant dTBP, which can substitute for the TFIID fraction for basal level activity^{9,19}. In lane 3, no additional proteins were added. Lanes 4 and 5 contain the control fraction, and lanes 6 and 7 contain ~ 1 and 3 ng TRF protein. *b*, TRF binds to the TATA sequence in a gel mobility shift assay but does not interact with TFIIB. Reactions in the odd-numbered lanes contain purified recombinant human TFIIB⁴. Lanes 2 and 3 contain dTBP; lanes 4 and 5 have the control fraction, and lanes 6 and 7 contain TRF. Both dTBP and TRF can bind to the TATA box, producing a protein-DNA complex (lanes 2 and 6). TFIIB cannot bind to DNA on its own (lane 1), but it can interact with TBP to produce a complex that migrates more slowly (compare lanes 2 and 3). Addition of TFIIB to reactions containing TRF produces no additional detectable complexes (compare lanes 6 and 7). **METHODS.** *a*, *Drosophila* embryo nuclear extracts were prepared and fractionated^{19,26}. TFIIB and TFIIE/TFIIF were fractionated through the S-Sepharose column, and RNA polymerase II was fractionated through the phosphocellulose step. The template for transcription was pBCAT¹⁸ containing the adenovirus E1B TATA box. Transcription was analysed by primer extension. *b*, Gel mobility shift assays were done as described¹⁹. Human TFIIB was provided by I. Ha and D. Reinberg. Recombinant *Drosophila* TFIIB (provided by S. Wampler and J. Kadonaga) was used in a similar experiment and gave identical results.

The identification of a TBP homologue that binds to the TATA element but not to TFIIB may allow further characterization of structural elements of TBP required for its interaction with TFIIB. A temperature-sensitive mutant of yeast TBP that supports transcription by RNA polymerase I but not by RNA polymerase II or III has been isolated²⁰; this mutant results from the substitution of serine for the proline present in all known TBP sequences, two residues before the 60-amino-acid repeated motif. In TRF, an isoleucine is found at the position of this proline (Fig. 1c). It remains to be determined whether this or other differences between TRF and TBP can account for the inability of TRF to function as a basal transcription initiation factor in the *in vitro* assay.

What might be the function of TRF? The highly restricted expression pattern of TRF is consistent with the idea that TRF is not a basal transcription factor. TRF might be a basal factor that mediates transcription initiation of a subset of genes in the CNS cells that express TRF (Fig. 2). In this model, TRF would have to interact not with TFIIB, but with a different protein that serves functions similar to those of TFIIB but which is not abundant enough to have been identified biochemically. Similarly, although TRF did not exert any detectable inhibition of basal transcription in the *in vitro* assay (data not shown), it is conceivable that TRF inhibits transcription of a specific subset of genes *in vivo* by competing with TBP for binding to the TATA box. Future biochemical characterization of those proteins that associate with TRF, as well as identification of potential downstream genes of TRF, for example those that show restricted expression patterns similar or identical to that of TRF, may further elucidate the function of TRF. Our results indicate that

TRF is a sequence-specific transcription factor which may regulate the expression of a subset of genes expressed in the CNS and testis. □

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How amino-acid insertions are allowed in an α -helix of T4 lysozyme

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STUDIES of extant protein sequences indicate that amino-acid insertions and deletions are preferentially located in loop regions¹, which has traditionally been explained as the result of selection removing deleterious mutations within secondary structural elements from the population. But there is no *a priori* reason to discount the possibility that insertions within secondary structure could either be tolerated until compensatory mutations arise, or have effects that are propagated away from secondary structure into loops. Earlier studies have indicated that insertions are generally tolerated, although much less well within secondary structure elements than in loop regions²⁻⁸. Here we show that amino-acid insertions in an α -helix of T4 lysozyme can be accepted in two different ways. In some cases the inserted amino acids are accommodated within the helix, leading to the translocation of wild-type residues from the helix to the preceding loop. In other cases the insertion causes a 'looping-out' in the first or last turn of the helix. The individual structural responses seem to be dominated by the maintenance of the interface between the helix and the rest of the protein.

One to three alanines were inserted at three different locations in the α -helix comprising residues 39 to 50 in T4 lysozyme (Fig. 1). Consistent with earlier studies^{3,4}, the insertions destabilize

the folded protein but not more so than do point mutations^{9,10} (Table 1).

Five insertion mutants have been crystallized and their structures determined and refined (Fig. 2). The insertion of a single alanine within the first turn of the helix in mutant N40-[A] causes residues Asn 40 and Ala 40a to 'loop out' into solvent, leading to a short interruption of the α -helix (Fig. 2a). Alanine 40a essentially replaces Asn 40 with its carbonyl oxygen hydrogen-bonded to the amide of Ser 44. The positions of Ala 41 and Leu 39 are practically unchanged. In contrast, in mutant N40-[SLD] (Fig. 2b), the inserted amino acids are accommodated by a shift in register that leaves the α -helix intact and leads to the displacement of the first two helical amino acids (Leu 39 and Asn 40) into the preceding loop (Fig. 3). This loop, which is already mobile in the cysteine-free pseudo wild-type (WT*) structure, now becomes disordered. A strikingly similar situation is found for mutant S44-[AAA], in which the insertion of three alanines in the middle of the helix causes a shift in register of three amino acids towards the N terminus (Figs 2d, 3). As a result, the side chains of the first six residues of the helix (except position 42) are changed relative to WT*. The main-chain atoms in the structures of N40-[SLD] and S44-[AAA] are essentially identical. Mutant S44-[AA] crystallized in a novel space group but has a structure similar to that of S44-[AAA] (Figs 2c, 3). Serine 44 occupies the former position of the buried Ala 42. Its buried hydroxyl does, however, make a hydrogen bond to the main-chain carbonyl of Asn 40 (ref. 11). In mutant K48-[HP], the insertion of the histidine and proline close to the C-terminal end of the helix results in the formation of a type 1 β -turn, a motif commonly found at the end of helices¹² (Fig. 2e). Lysine 48 and the carbonyl oxygen of Ile 50 remained essentially unchanged, whereas Ala 49, the rest of Ile 50, and the two inserted amino acids, are located in the β -turn.

Two-dimensional NMR analysis of ¹⁵N-labelled mutants¹³ shows that the backbone conformations of N40-[AAA] and K48-[AA] are, respectively, similar to N40-[SLD] and K48-[HP]. NMR also shows that N40-[AA] has a translocation

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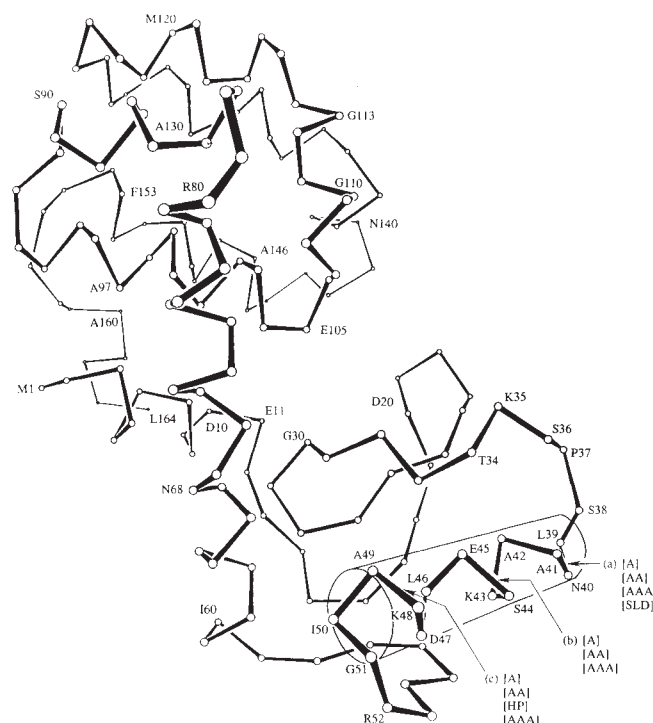


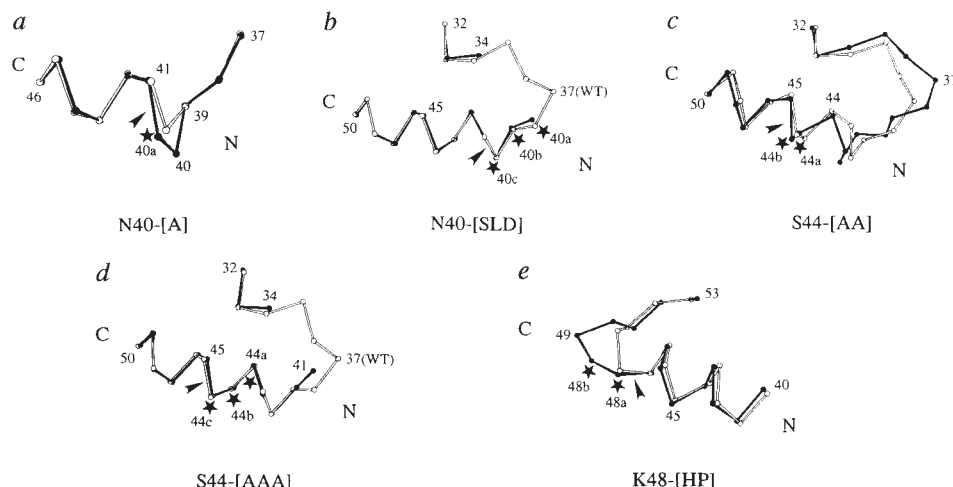
FIG. 1 Backbone of T4 lysozyme showing the sites of amino-acid insertions (a) between Asn 40 and Ala 41, (b) between Ser 44 and Glu 45, and (c) between Lys 48 and Ala 49 in the α -helix 39-50. This amphipathic helix packs against the small hydrophobic core of the N-terminal domain of the protein and is flanked by a solvent-exposed irregular loop (residues 35-38) at its N terminus and another loop (residues 51-57) at the C terminus. Insertion mutants were constructed on a cysteine-free pseudo wild-type T4 lysozyme (WT*) background²² by site-directed mutagenesis²³ using oligonucleotides containing inserted alanine codons flanked by 12-15 nucleotides. Mutants were selected, cloned and purified as described²⁴. In independent experiments, further mutagenesis was used to change the inserted amino acids randomly and variants with increased stability compared to the alanine-insertion background were selected using a halo assay²⁵. These include mutants N40-[SLD] and K48-[HP], which are germane because their crystal structures are available. In addition, Asn 40 was replaced by leucine in mutant N40-[A], giving the mutant N40L-[A], and Ser 44 was replaced by alanine in mutant S44A-[AA]. These mutants are all more stable than their respective parents (Table 1).

similar to N40-[AAA] and N40-[SLD], and that the α -helical conformations of the inserted amino acids seen in the crystal structures of S44-[AA] and S44-[AAA] also occur in solution.

A dominant factor in determining the nature of the structural response to a given insertion appears to be the maintenance of the buried hydrophobic interface between the α -helix and the remainder of the protein. For example, the calculated solvent accessibility¹⁴ of residues involved in this interface does not change when compared with wild type. Also, calculations¹⁵ with a probe radius of 1.2 Å did not detect any cavity in the interface of any of the crystal structures. (A 1.0 Å probe showed a small

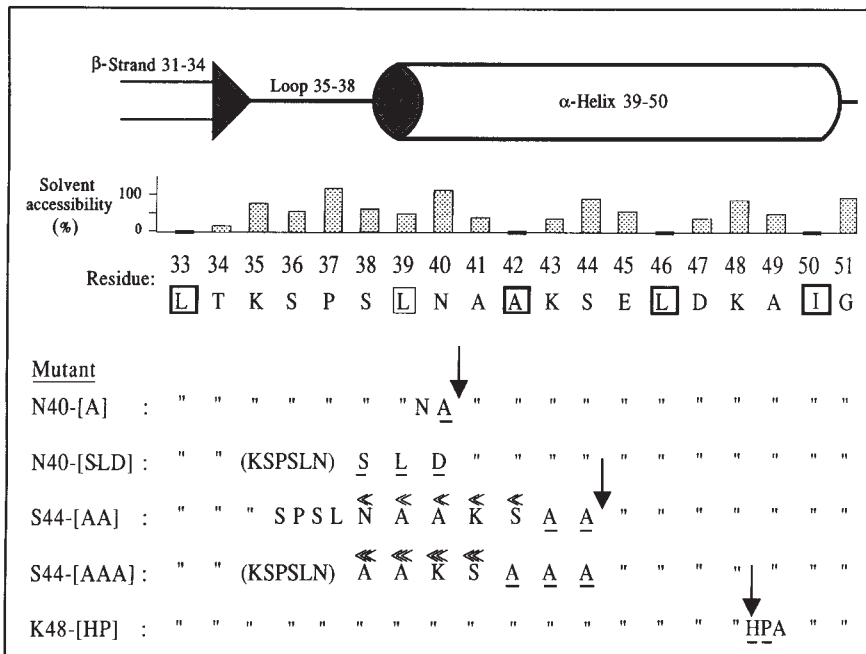
cavity (6.6 Å³) for S44-[AA], which was expected to be slightly destabilizing¹⁰.) Small hydrophobic crevices on the surface of the helix were found in cases where substitutions in the helix resulted from the register shift (for example, L39A and K43A in S44-[AAA]; Fig. 3). These mutations are destabilizing as well⁹. Several buried residues in the helix serve as anchors and strongly resist translocation (Fig. 3). Isoleucine 50 is one such anchor residue, maintaining its location in spite of a nearby insertion between residues 48 and 49. Leucine 46 in the middle of the helix is a second anchor, maintaining its position in all variants. Alanine 42 possibly serves as a third anchor. Translo-

FIG. 2 a-e, Superposition of C α -positions from the crystal structures of five different insertion mutants (represented by filled lines) on WT* lysozyme (bonds represented by double lines). Superpositions are by least-squares, including all main-chain atoms within the amino-terminal domain (residues 1-60), except residues directly influenced by the insertion. Average discrepancies were 0.14-0.35 Å for isomorphous structures and 0.45-0.50 Å for non-isomorphous ones. Inserted amino acids are labelled using the suffix a, b or c, depending on their position relative to the preceding residue, and are marked with stars. Arrowhead shows the site of the insertion. Letters N and C indicate the N and C termini of the α -helix. Disordered residues 35 to 40 in the structures of N40-[SLD] and S44-[AAA] are omitted (see text). a, N40-[A]; b, N40-[SLD]; c, S44-[AA]; d, S44-[AAA]; e, K48-[HP]. Crystals isomorphous to WT* lysozyme (space group P3₂2₁; resolution limits 1.74 Å, 1.9 Å and 2.1 Å) were obtained for N40-[A], N40-[SLD] and S44-[AAA] using conditions previously described^{10,26}. Non-isomorphous crystals of S44-[AA] and K48-[HP] suitable for data collection were grown using a rapid screening procedure²⁷. S44-[AA] crystallized in space group R32 and its structure to 2.8 Å resolution was determined by molecular replacement. Mutant K48-[HP] crystallized in space group P2₁



isomorphously with the mutant S44F, whose structure had been determined (M. Blaber, X.-J. Zhang and B.W.M., unpublished results), making it relatively straightforward to refine the structure of K48-[HP] at 2.0 Å resolution. Details of the structure determinations will be given elsewhere. Coordinates have been deposited in the Brookhaven Data Bank. No definitive crystallographic or NMR structural data have been obtained so far for mutants S44-[A], K48-[A] and K48-[AAA].

FIG. 3 Summary of structural changes observed in insertion mutants of T4 lysozyme. Amino acids are identified by the one-letter code. Inserted amino acids are underlined and vertical arrows indicate the site in the wild-type sequence where the insertion was made. The alignment of each mutant structure relative to WT* is based on the correspondence of the crystal structures (Fig. 2). Left-pointing arrowheads indicate residues translocated two or three positions towards the N terminus (no translocations towards the C terminus were seen). Residues that have substantially different structures and 'loop out' in the mutant structures are identified but do not align with wild type (for example, Asn 40 and Ala 40a of N40-[A]). Disordered residues are shown in parentheses. Amino acids that are unchanged and essentially superimpose in the mutant and wild-type structures are indicated (") (such as residues 33-39 and 41-51 in N40-[A]). Residues with essentially the same backbone structure as WT*, but with substituted amino acids, are identified and align with their wild-type counterparts (for example, Ser40a, Leu 40b and Asp 40c in N40-[SLD]). Residues Leu 33, Ala 42, Leu 46 and Ile 50 (bold boxes) are buried or nearly buried in the native structure and are presumed to act as anchors. Leucine 39 (boxed) is partly buried and appears to act as a secondary anchor. Solvent accessibility (for WT* as well as the insertion mutants) was calculated¹⁴ as a ratio of the side-chain area accessible to solvent relative to the same area in an extended model peptide of identical sequence²⁸.



cations past this site are observed in S44-[AAA] and S44-[AA], although in the first case another alanine is substituted and in the second case the small (but polar) residue serine is allowed. Although serine is tolerated at site 42 in S44-[AA], it clearly is destabilizing. If the serine is replaced by alanine, as in mutant

TABLE 1 Thermal stabilities of insertion mutants

Lysozyme	ΔT_m (°C)	ΔH (kcal mol ⁻¹)	$\Delta \Delta G$ (kcal mol ⁻¹)	Structural response
N40-[A]	-7.9	97	-2.8	Looping out
N40L-[A]	-3.9	122	-1.4	ND
N40-[AA]	-3.0	121	-1.2	Translocation
N40-[AAA]	-4.8	116	-1.7	Translocation
N40-[SLD]	-2.0	127	-0.8	Translocation
S44-[A]	-11.8	55 [†]	-3.5	ND
S44-[AA]	-9.9	81	-3.3	Translocation
S44A-[AA]	-4.4	114	-1.7	Translocation
S44-[AAA]	-6.0	106	-2.2	Translocation
K48-[A]	-11.4	65 [†]	-3.5	ND
K48-[AA]	-10.5	75 [†]	-3.4	Looping out
K48-[HP]	-7.1	104	-2.5	Looping out
K48-[AAA]	-14.8	52 [†]	-4.2	ND

Insertion mutants are denoted by the amino acid (in single-letter code), with its position, that precedes the inserted amino acids given in brackets (N40-[AAA], for example, indicates a triple alanine insertion following Asn 40). Thermal stabilities were determined^{19,20} at pH 5.4 in 0.10 M NaCl, 0.01 M sodium acetate. In this solvent, the pseudo wild type WT* melts at 65.2 °C with a ΔH of 136 kcal mol⁻¹. Data were analysed²¹ to determine the temperature, T_m , at which the protein is half-folded and the enthalpy of unfolding, ΔH , at the melting temperature. The average error for T_m was ± 0.2 °C, and for ΔH , ± 6 kcal mol⁻¹. ΔT_m is the T_m of the mutant protein minus that of WT*. Isothermal differences between the free energy of unfolding of the mutants and that of WT* ($\Delta\Delta G$) were calculated²⁰ with a constant value of ΔC_p of 4.5 kcal deg⁻¹ mol⁻¹ at 53 °C. This isotherm was chosen because the behaviour of some mutants (ΔH marked with a double dagger) departs from that expected for a two-state system in that the absolute values of the apparent ΔH are low. We estimate the error to be ± 0.4 kcal mol⁻¹ for the $\Delta\Delta G$ values of all the mutants. The mutations have little if any effect on catalytic activity.

S44A-[AA], the overall destabilization of the protein is reduced from 3.3 kcal mol⁻¹ to 1.7 kcal mol⁻¹ (Table 1). Leucine 39 is partly buried in a hydrophobic crevice and this site also acts as a subsidiary anchor. Translocations past this site are allowed when the substituted residue is hydrophobic (Ala) (in N40-[AA], N40-[AAA], S44-[AA] and S44-[AAA]), but not when the potential substitution would be hydrophilic (Asn 40 in N40-[A]) (Fig. 3). This hypothesis is confirmed by the observation that the replacement of Asn 40 with leucine (in N40L-[A]) increases the stability of the protein by 1.4 kcal mol⁻¹ (Table 1). The apparent preservation of the interface also agrees very well with principles found from the analysis of the structural evolution of proteins¹⁶.

Insertions that cause looping out within the α -helix tend to be more destabilizing (average 2.9 kcal mol⁻¹) than insertions that cause translocation along the helix (average 1.8 kcal mol⁻¹) (Table 1). This suggests that translocation will be preferred unless resultant unfavourable interactions exceed the energy cost of looping out. Shifts in register have also been proposed on the basis of insertion mutants in *Staphylococcus* nuclease⁴ and bacteriorhodopsin⁶.

The observed translocations do not result in an increase in the length of the helix, suggesting that helix length is determined by the extent of the tightly packed interface with the rest of the protein and is not defined by the amino-acid sequence at the beginning and end of the helix¹⁷.

Our results provide some new insight into protein sequence comparison based on known three-dimensional structures. In such studies^{1,18}, the overwhelming majority of amino-acid insertions are found in loop regions. This suggests that insertions (or deletions) perturb or destroy the precise interdigitation of residues at interfaces and lead to an unacceptable destabilization of the protein. Here we find that some insertions can be accommodated in the middle of a helix (as in S44-[AAA]) and cause an extension of a preceding loop which is more than 10 Å away. Subsequent point mutations (as in N40-[SLD]) can regain lost stability. Because the revertant N40-[SLD] restores wild-type amino acids within the α -helix (Fig. 3), the net effect is to replace the initial insertion, which was within the helix, with a substitution within the helix plus a different insertion in the

loop at the end of the helix. On the basis of comparison of existing structures, it may be very difficult, if not impossible, to differentiate between insertions that occurred in loops, as opposed to insertions that originally occurred in helices but were propagated into neighbouring loops. □

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ERRATUM

Evolution and environment in the Hominoidea

Peter Andrews

Nature **360**, 641–646 (1992)

IN this Review Article, in the figure in Box 4 on page 644, the labels 'Orang' and 'Human' in the diagram labelled "Classification at family, subfamily and tribe level of the Hominoidea" were inadvertently transposed.

CORRECTIONS

Molecular and genetic damage in humans from environmental pollution in Poland

Frederica P. Perera, Kari Hemminki, Ewa Gryzbowska, Grazyna Motykiewicz, Jadwiga Michalska, Regina M. Santella, Tie-Lan Young, Christopher Dickey, Paul Brandt-Rauf, Immaculata DeVivo, William Blaner, Wei-Yann Tsai & Mieczyslaw Chorazy

Nature **360**, 256–258 (1992)

IN Table 1 on page 257, the *P* values for the comparisons of PAH-DNA adducts between the 'Exposed winter' (EW) to 'Control summer' (CS) and 'Exposed summer' (ES) to CS are reversed. The correct *P* value for the comparison of EW to CS is 0.012 and that for the comparison of ES to CS is 0.078, as expected from the means in the table. The correct *P* values further strengthen the finding of an effect of air pollution on DNA adducts.

Cloning and characterization of a gene that regulates cell adhesion

W. E. Pullman & W. F. Bodmer

Nature **356**, 529–532 (1992)

SHOWN below is a revision of the nucleotide sequence and inferred amino acid sequence for a cellular adhesion regulatory molecule, the gene for which is now known as CMAR (CAR in this letter). The authors acknowledge helpful information received from Drs Alejandro Aruffo and Po-Ying Chan of Bristol-Myers Squibb, Pharmaceutical Research Institute, Seattle, USA, and H. Durbin (ICRF) in making this correction.

CMAR

```
GCATGGAACACTTCGAGTTCGCCAGGGTTATAGACAGTCGTTCCCGAGTGTGG      51
CTGAGGCCACCCAGGACGAGGAGCATTACAGCTCCAAACAGACCCCTGT          102
TCATGCCGACGCTGCACGACGCGCCCGAGTTCCTCTGGCTCCCTCGGAATG      153
      _ _ _ _ _ M _ _ _ _ _
CTAAGGGGATCGGACATGAAAGGACCCCTGTGAGCCGATTGTCTCTATCTCCA      204
L R G S D M K G P C E P I V L S P
_ _ _ _ _
GCGGCCCTGTATCCAGCTCACTCATCAATGGGGCCAGTCAGGCCAGGCA      255
A A L S S S S L I N G A S Q A Q A
_ _ _ _ _
CTGGGCTCCGGAGGACTCACCAGTCCGCCCTGCTGCCATGTGGAGTGGTGC      306
L G S G G G L T T A P C C H V D W C
_ _ _ _ _
AAGTTGAGGACTTCTTGTCTGGTCTAGTCACGCATGCAGTGTGGGGATGCC      357
K L R T S C W S S H A C S V G D A
_ _ _ _ _
TTGGTTTTCAGTCTCTGAGATTGTTGAGATACITTTACTAATAAACTGTG      408
L V F T A L R I V E I L Y
_ _ _ _ _
TAGTTGGAAAAA          429
```

myristoylation site

tyrosine phosphorylation site

Multiple-sheathflow capillary array DNA analyser

Hideki Kambara and Satoshi Takahashi

A sensitive, high-throughput fluorescent DNA analyser has been developed that uses sheathflows of buffer around each element in an array of gel capillaries.

DNA analysis is one of the key technologies in the life sciences and various automated DNA analysers are now commercially available that are designed to assist in this process^{1,2}. With the start of the human genome project, however, it would be desirable to develop an analyser that is more sensitive and that has a higher throughput than the instruments available at present. The human genome is so large that to determine and make a comparative study of the base sequence of the whole genome will require a DNA sequencer with a throughput of at least one million bases a day³.

In addition to these efforts to catalogue the base order of the human genome, sequencing data are providing interesting new information from a general biological standpoint. For example, functional mRNA species that are identical within tissues are being studied⁴. Analysing the DNA or mRNA from a single cell — especially the mRNA abundance at various stages in the life of a single cell — would provide information about such biological phenomena as differentiation, specialization, proliferation and senescence. Even if the sensitivity of analysis was not so high and nucleic acid from several hundred cells was required, important information could still be obtained.

We can, for example, make DNA probes that have various migration speeds in gels and that can be assigned to certain mRNA species. With a highly sensitive DNA analyser, it would be possible to analyse mRNA abundance in tissues at various life stages by analysing the DNA probes hybridized to mRNA. The sensitivity of the fluorescent DNA sequencers available at present, however, is only 10^{-17} to 10^{-18} mol per band^{5,6}. This sensitivity is so low that very large amounts of DNA are required for analysis. An analyser is needed, therefore, that is not only sensitive but that also achieves a high throughput by analysing many kinds of probes at a time.

Capillary gel electrophoresis is a method that promises to provide high sensitivity and rapid operation^{7,8}, and a capillary array could be used to make a high-throughput DNA analyser. Mathies *et al.* have reported a capillary array electrophoresis apparatus that uses a confocal fluorescence detection method⁹, and our group has developed an ultrasensitive DNA analyser that uses side-entry laser irradiation¹⁰.

Here we report the characteristics of and prospects for a new capillary array electrophoresis unit that can be adapted to the ultrasensitive DNA analyser by using a multiple-sheathflow technique.

Multiple-sheathflow system

In the fluorescence detection system, the detector is orientated perpendicular to the excitation light as this geometry minimizes background scattering. Although this geometry is commonly used in single capillary experimental set-ups, the main question that we had to answer in developing this analyser was how it could be used with a capillary array.

Figure 1 illustrates schematically how this has been achieved by removing the capillary walls at the irradiation region where the excitation light is scattered and refracted. The electrophoresis tracks consist of upper and lower capillary arrays connected by a flow cell filled with buffer solution. The upper capillary array consists of 20 capillary tubes filled with gel and lined with a 0.5-mm pitch. The inner (i.d.) and outer diameters (o.d.) of the capillary tubes are 0.1 mm and 0.2 mm, respectively. The lower capillary array consists of 20 open capillary tubes (0.2-mm i.d., 0.3-mm o.d.). The capillary arrays are aligned, 1 mm apart, so that the axes of the upper and the lower tubes coincide. The buffer solution ($1\times$ Tris-Borate-EDTA) in the flow cell flows into the lower capillary tubes, producing a sheathflow around each capillary tube. DNA fragments eluted from the upper capillary tubes flow into the lower capillary tubes together with the buffer flow. The 6-mW He-Ne excitation laser (594 nm) irradiates DNA fragments in the sheathflow region 0.5 mm below the ends of the upper capillary tubes. A cylindrical lens system gathers the fluorescence from the irra-

diated line, and the fluorescent image is recorded on a two-dimensional detector (a cooled CCD camera coupled with an image intensifier). The length of the observed region can be varied from 20 mm to 120 mm by changing the distance between the irradiated region and the camera (a typical length in the capillary array mode is 40 mm). The labelling fluorophore is Texas Red (Molecular Probes, Inc., Eugene, Oregon) ($\lambda_{ex} = 594$ nm, $\lambda_{em} = 615$ nm). Although the experiment reported here used a single colour, this system can be easily modified for multicolour detection by placing an image-splitting prism in front of the camera¹¹.

Characteristics and prospects

Because this system is easy to set up and has no moving parts, its operation is stable. In the slab-gel mode, the detection sensitivity for Texas Red-DNA is 3×10^{-13} M (signal-to-noise ratio of ~ 2). When detecting

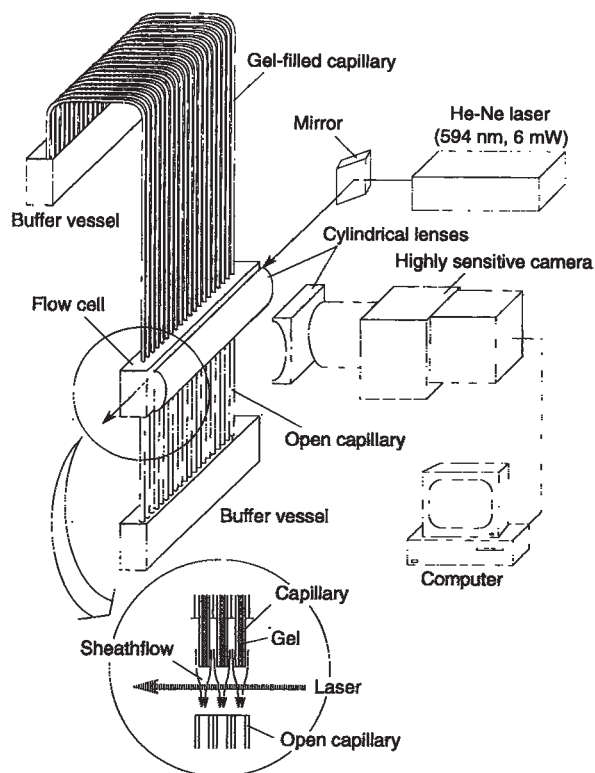


FIG.1 Multiple-sheathflow gel-capillary array apparatus.

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double-stranded DNA fragments, which have fluorophores at their 5' terminus and therefore have two fluorophores, the minimum quantity that could be detected was 3×10^{-20} mol per band (signal-to-noise ratio of ~2) when the injection volume was $0.5 \mu\text{l}$. In the capillary array system, on the other hand, the background fluorescence decreased by one order of magnitude because there were no scattering materials (gel and glass surfaces) in the irradiated region. The background fluorescence from the buffer solution was as great as that from 1×10^{-12} M of Texas Red-DNA. Although the background fluctuation was ± 3 per cent, which is a little bit higher than that obtained with a slab gel, flowing 1×10^{-12} M of Texas Red-DNA revealed that the limit of sensitivity was 1×10^{-13} M (signal-to-noise ratio of ~2).

In capillary array systems, it is difficult to estimate the quantity injected as samples are injected electrically. Assuming the sample volume to be 10 nl, we estimate the minimum quantity detectable to be 10^{-21} mol per band. This sensitivity is high enough to investigate mRNA abundance from hundreds of cells (if there is an appropriate method for introducing the sample). Sensitivity can be improved by using a narrower capillary and a detector with a higher spatial resolution. Even the detection of single molecules should be possible in the capillary array system in the future.

For higher throughput, it is useful to have many capillaries in a bundle. In addition, narrow lane spacing is also preferable for the purposes of detection because the overall fluorescence collecting yield of the present cylindrical lens system and scanning system is inversely proportional to the length of the region of measurement. The present lane spacing of 0.5 mm is greater than that in the most tightly packed array (0.2-mm pitch). Although the region of measurement was as much as 40-mm long, simultaneous irradiation of all the lanes and the cylindrical lens system provided a fluorescence collecting efficiency high enough to detect trace amounts of DNA.

Besides detecting trace amounts of DNA, high throughput is especially important for DNA sequencing. This requires many migration lanes and rapid electrophoresis. As this system has no moving parts, the data sampling period can be reduced to less than 0.1 s. The present system can use more than 100 capillaries by increasing the observable region without losing sensitivity. Figure 2 shows part of the DNA fragment spectra for 'A' base DNA fragments of M13mp8. The base-reading speed was 300 bases per hour. Because the migration time of a given DNA fragment varies from capillary to capillary, four-colour detection is advantageous for capillary array DNA sequencing. This system can be converted to four-colour detection by the addition of an image-splitting prism and colour filters. The resolution and migration time depended on the quality of

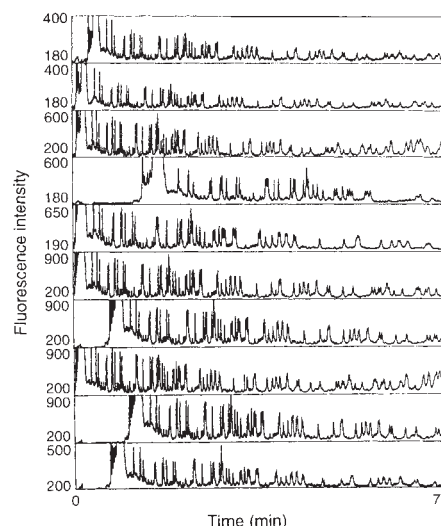


FIG.2 DNA fragment spectra of 'A' base DNA fragments of M13mp8 obtained by gel-capillary array analysis (10 capillaries).

the capillary gel, which is not reproducible at present. Although with DNA fragments shorter than 200 bases, the band resolution obtained with a gel capillary was better than that obtained with a gel slab, the upper limit of one-base resolution for long DNA fragments was similar to that obtained with a slab-gel system¹².

Gel-filled capillaries are, nevertheless, useful for analysing long fragments because it is much easier to handle a long capillary gel than a long slab gel. When a 3-m long capillary was used, one-base difference could be resolved in fragments of up to 800 bases long.

This highly sensitive capillary array system shows promise for rapid, high-throughput DNA sequencing, direct sequencing from colonies without cloning, and for the rapid analysis of mRNA abundance within a single cell or tissue. □

Hideki Kambara and Satoshi Takahashi are in the Central Research Laboratory, Hitachi Ltd, Kokubunji, Tokyo 185, Japan. For more information, fill in reader service number 100.

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New twists in DNA technology

Research tools for the molecular biologist featured this week include paints for the detection of human chromosomes 11 and 12, a kit for isolating differentially expressed genes, gel chambers, gels and other paraphernalia.

FINNZYMES Oy has developed a new **thermostable DNA polymerase** called Dynazyme, which is derived from *Thermus brockianus* FZ500 (Reader Service No. 101). Designed to tolerate high temperatures, Finnzymes says that Dynazyme has a half life of 2.5 h at 96 °C. The associated enzyme activities include 5' → 3' DNA polymerase activity and 5' → 3' exonuclease activity. The company also claims that Dynazyme provides high fidelity upon DNA synthesis, yielding a two-fold lower error frequency than, for example, *Taq* polymerase (when measured using the forward mutational assay). The enzyme lacks 3' → 5' exonuclease activity — a characteristic that should make it easy to use, generally requiring no further optimization of reaction conditions. It is supplied with optimized reaction buffers and is available in 100- and 500-unit packages.

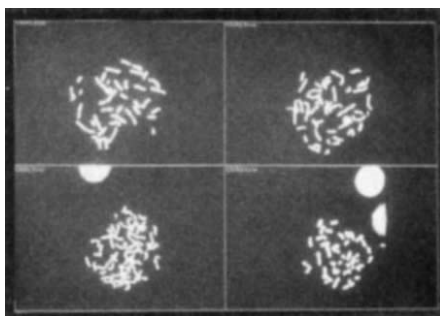
The first full-length listing for Santa Cruz Biotechnology's novel antibodies focused on **signal transduction research** has been released by New Brunswick Scientific — the company's distributor in the United Kingdom (Reader Service No. 102). Many of these new products are available only from Santa Cruz, for example, ImmunoCruz Systems for protein detection and identification and TransCruz Gel Supershift reagents for gene transcription research. Other products include *c-fos*(4) rabbit polyclonal IgG, the *trk* gene family of cell membrane receptors and p53(DO-1) mouse monoclonal antibody IgG.

Promega's Packagene **lambda DNA packaging system** uses an *Escherichia coli* C bacterial host to produce the extract, unlike the standard two-strain packaging systems based on *E. coli* K12 (Reader Service No. 103). As *E. coli* C contains no restriction systems, libraries produced in *E. coli* C are unbiased by restriction of genetic material. Libraries with efficiencies of greater than 10⁷ pfu µg⁻¹ vector DNA are obtained routinely, says Promega.

Boehringer Mannheim's 5-Bromo-2'-deoxyuridine **labelling and detection kit III** is a standard cell ELISA in a microtitre plate format (Reader Service No. 104). It allows for the complete replacement of the classical ³H-thymidine-based cell proliferation assay and can be used for both the analysis of adherent cells and cells in suspension. The avoidance of radioisotopes is designed to increase safety and provide added convenience. 5-Bromo-2'-deoxyuridine (BrdU) is incorporated into freshly

synthesized DNA and is monitored as a parameter for DNA synthesis and cellular proliferation. Cells that have incorporated BrdU into DNA are detected using a monoclonal antibody to BrdU and an enzyme-conjugated second antibody. The company also offers the cell proliferation kit I (MTT) and the cell proliferation kit II (XTT). Both kits constitute colorimetric assays that use the tetrazolium salt MTT or XTT to report cell proliferation, viability and cytotoxicity. MTT and XTT indicate cellular activity by serving as substrates for mitochondrial dehydrogenases. Their metabolism results in the formation of formazan dyes, which are easily quantitated using a standard multiwell ELISA reader. MTT- or XTT-based cell proliferation and viability assays are used for the quantitative determination of cellular proliferation and activation, for example, in response to growth factors and cytokines. They are also used for the quantification of antiproliferative or cytotoxic effects (for example, those mediated by tumour necrosis factor-α). In cancer research, the MTT assay is used for quantification of *in vitro* chemosensitivity of tumour cells and for screening compounds.

Cambio has expanded its range of **paints for the detection of human chromosomes** with the addition of numbers 11 and 12 (Reader Service No. 105). This means that paints are now available for every chromo-



Chromosome numbers 13, 15, 18 and 21 visualized using the Cambio chromosome painting system.

some (including X and Y) with the single exception of number 10. The company anticipates that this remaining paint will be available shortly. The paints are designed to provide even coverage of the chromosome arms, unlike paints based on clone libraries. Visualization is normally achieved using the well-established biotin-avidin or biotin-streptavidin detection systems, allowing the use of any fluorochrome coupled to avidin

or streptavidin. The paints can also be used with several other detection systems, including horseradish peroxidase, alkaline phosphatase and gold. Several kits are available with chromosome counterstains to provide fluorescence reverse banding simultaneously with a chromosome-specific signal (FITC and/or Texas Red). This makes them compatible with both epifluorescence and laser-scanning confocal microscopy. The paints are supplied ready to use in hybridization buffer.

■ Kits

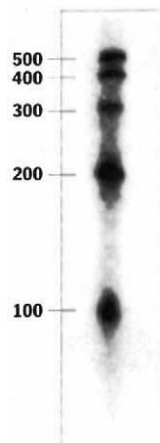
GenHunter has introduced RNAmapping kit, a complete system for **differential display of mRNAs** (Reader Service No. 106). The kit, which is based on the differential display technique described by Peng Liang and



mRNA differential display kit for isolating differentially expressed genes.

Arthur Pardee (*Science* **257**, 967–971; 1992 and *Cancer Res.* **52**, 6966–6968; 1992), is intended to be a useful tool for identifying and cloning differentially expressed genes responsible for a host of normal biological processes such as cell differentiation and pathological states such as cancer and genetic diseases. The method involves reverse transcription of mRNAs using anchored oligo-(dT) primers followed by cDNA amplification in the presence of a second short arbitrary primer. cDNA fragments corresponding to the 3' tail of mRNAs are distributed on a denaturing polyacrylamide gel. By changing primer combinations, GenHunter says that nearly all the mRNA species in a cell may be visualized. Each US\$235 kit contains all the possible anchored oligo-(dT) primers and five arbitrary 10-mers — enough to display ten mRNA samples in all primer combinations. The protocol includes mRNA differential display, elution and reamplification of cDNA probes for subcloning or northern blot analysis.

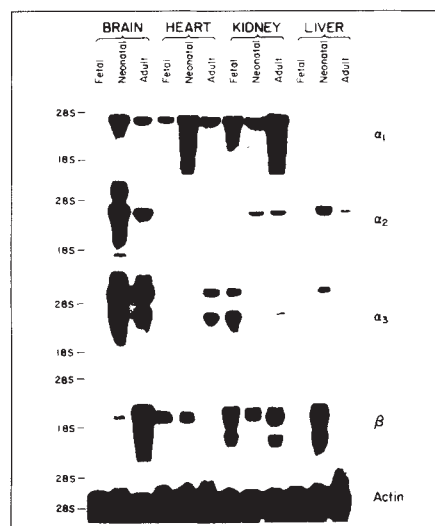
Ambion has recently released an RNA Century Marker Template Set consisting of a set of five different **linearized plasmid template** (Reader Service No. 107). The set yields five RNAs with lengths of 100, 200, 300, 400 and 500 bases after transcription with T7 RNA polymerase. These RNA fragments may be easily labelled during transcription with radioisotopes, biotin or other nonradioactive ligands. These markers are intended for sizing RNAs for ribonuclease protection assays, S1 assays, primer extension assays, and other precise studies of RNA structure. By providing linearized templates for the researcher, he or she gains more flexibility in determining labelling schedules and procedures. Unlabelled RNA Century Size Markers are also available separately. These RNA fragments can be visualized by staining with ethidium bromide.



RNA Century marker template set.

■ Blotting methods

Northern blots for RNA transcript analysis and developmental transcript analysis have been added recently to Bios Laboratories' BIOSMAP product line (Reader Service No. 108). These products allow the user



Rat developmental analysis blot containing total RNA isolated from brain, heart, kidney and liver extracted from fetal, neonatal and adult tissues. This blot was hybridized with probes that identify different rat sodium-channel genes. The sodium-channel genes are indicated on the far right. An actin probe was also hybridized to this blot to show that each lane contains equivalent amounts of RNA. (Courtesy of Janet Emmanuel, Yale University.)

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to determine rapidly the pattern of gene expression in eight tissues from rat, rabbit, mouse, human or monkey. Researchers can also elect to study the expression in four different tissues at three developmental stages in either rats or humans. BIOSMAP northern blots contain total RNA isolated from brain, heart, kidney, liver, lung, spleen, intestine (stomach in human and rat tissue), and skeletal muscle. Bios says that these tissues were selected to create an expression screen as an interface between gene localization and functional studies. The source of these RNAs are: mouse (C57Bl/6J, adult), rat (Wistar, two-day-old, adult), rabbit (New Zealand White, adult), monkey (rhesus, adult), human (10-week gestation, 20-week gestation, adult). Three product formats are available: tissue, developmental and custom. The standard tissue blot includes brain, heart, kidney, liver, lung, spleen, intestine (stomach for human or rat), and skeletal muscle from one developmental stage of one species. The developmental blot arrays four standard rat or human tissues at each of three developmental stages. The researcher selects the specific tissues. Bios custom blots, on the other hand, can be prepared to meet specific research needs.

LumiGLO western blot HRP systems available from Kirkegaard & Perry Laboratories provide all the reagents you need for rapid, convenient, **nonisotopic western blotting** (Reader Service No. 109). These systems are designed to offer the enhanced sensitivity of chemiluminescent detection with the safety and stability of an all-liquid system. The company says that peroxidase-labelled, affinity-purified conjugates are optimized for maximal signal-to-noise performance. A two-component luminol-based substrate is included, in addition to wash and block solutions. Results are recorded using standard X-ray film, providing a permanent hard copy. Results can also be developed on film using a camera luminometer. These systems are intended to work well on nitrocellulose, PVDF and nylon membranes, and include an antibody conjugate of your choice for the detection of primary antibodies made in mouse, rabbit or human. Kirkegaard & Perry says that reagents are stable for 12 months. Kit instructions and a troubleshooting guide are included.

■ PCR

PCR Optimizer is a new kit from Invitrogen that is designed to allow researchers to **determine the optimal amplification conditions** for each primer/template combination in order to maximize the quantity and quality of PCR products (Reader Service No. 110). Invitrogen says that the kit permits rapid evaluation of several critical reaction conditions to identify the optimal conditions for each amplification reaction. The kit provides reagents for a three-stage evaluation of 32 different buffer conditions

and an easy-to-follow matrix to determine rapidly the best amplification conditions in the fewest number of steps. The parameters that are evaluated include the pH level, magnesium concentration and the presence or absence of dimethylsulphoxide. Sufficient reagents (including all buffers and dNTPS but excluding *Taq* polymerase) are included for 100 reactions.

The new PCR Purity Plus kit from AT Biochem offers a means of **verifying PCR product purity** (Reader Service No. 111). AT Biochem says that the gel offers good resolution, a wider separation range and enhanced sensitivity over specialty-grade agarose and acrylamide. The PCR Purity Plus gel is supplied in a kit that allows the user to check quickly and conveniently the amplified DNA samples. Included in the kit are ten disposable cassettes (compatible with all commonly used mini-vertical electrophoresis apparatus), two reusable 14-well combs, a 50–2,500-base pair molecular weight marker and a triple-dye loading buffer. Each component of the kit is also available individually. PCR Purity Plus is the latest member of the HydroLink family of high-performance gels.

The new 6-mm COPYmRNA Capture membranes from RNA Lab, which are specifically configured for reverse transcriptase-polymerase chain reaction (RT-PCR), can be used to **capture mRNA directly from cell or tissue lysates in chaotropic salts** in 30 min (Reader Service No. 112). No special equipment is needed. The COPYmRNA Capture membranes should be useful to researchers studying mRNA by sequence amplification.

■ DNA synthesis

Ana-Gen Technologies introduces Trityl-Tech, an **online trityl monitor** that performs real-time coupling efficiency analysis of automated DNA/RNA synthesizers (Reader Service No. 113). The system incorporates an end-point method to measure the DMTr cation concentration in the effluent collected at each detritylation step of the synthesis cycle. The effluent from the trityl port of the synthesizer is collected in glass cuvettes (1-cm path length), mixed and the absorbance is determined photometrically.



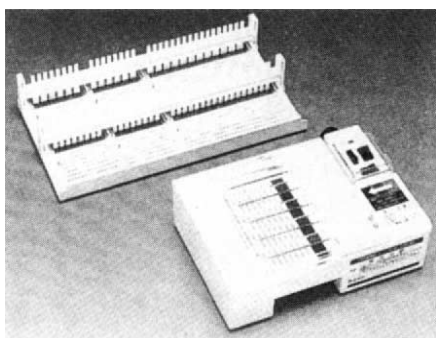
Online coupling efficiency monitor.

Real-time coupling efficiencies can be monitored at each step of the synthesis cycle; this enables the user to interrupt synthesis if the coupling efficiency drops below acceptable levels, with resultant cost savings in the consumption of expensive reagents, Ana-Gen says. These data are collected by the computer and coupling efficiency, stepwise yield and the theoretical amount of crude is automatically calculated and stored for each cycle. Hard copies of these data in the form of bar charts and tables are printed at the end of each synthesis. The collected and calculated data are stored as ASCII files and can be imported into popular database, spreadsheet or word processing software packages for further analysis.

Automated synthesis of DNA is a routine process today and requires only a few hours to assemble an oligomer 40 bases long. Post-synthesis workup, however, can take much longer than the actual chain assembly. Most deprotection methods require treatment of the oligo at 55 °C for 6 h or 24 h at room temperature to remove completely the protecting group, which can be a significant and unwelcome delay. The new Expedite fast **DNA synthesis reagents** from Millipore are designed to speed up DNA production and improve quality (*Reader Service No. 114*). This is accomplished by using the new base-protecting group, tert-butylphenoxyacetyl, which has been developed by Millipore. The company says that this new protecting group is stable to the reaction conditions used in synthesis but very labile to the ammonium hydroxide used in deprotection. The result, says Millipore, is dramatically shortened deprotection times — only 15 min at 55 °C or 2 h at room temperature. Expedite reagents, compatible with multiple synthesis chemistries, allow researchers to synthesize a full range of base-labile and modified nucleosides, in addition to phosphorothioated oligos. Millipore says that oligos synthesized with Expedite chemistries retain 99 per cent base integrity and have proven effective in biological studies.

■ Gel boxes

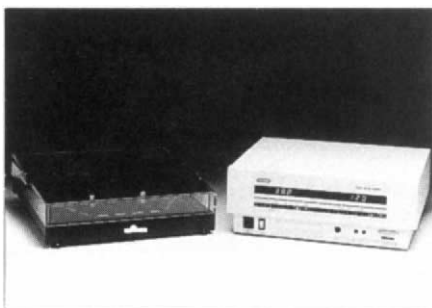
Mupid-2 is the new **gel electrophoresis system** for DNA/RNA (agarose) or protein (polyacrylamide) gel electrophoresis from



Mupid-2 with its own built-in power supply.

DNA Technologies (*Reader Service No. 115*). The system comes complete with six minigel migration trays, combs, a gel casting tray and built-in power supply that has an output voltage of 50 or 100 V. The gel marker sets can be used to prepare four small (52 × 60 cm) and two large (107 × 60 cm) gels simultaneously with the help of two different size combs. Gels are prepared without any taping. The company says that the polarity of the migration can be changed by the simple flip of a switch. Mupid-2 is available at an introductory price of US\$495.

The new CHEF DR III **pulsed-field electrophoresis system** from Bio-Rad combines the variable-angle, electronic switching capability of the PACE technology from the CHEF Mapper XA system with the reliability and performance of the CHEF DR II system (*Reader Service No. 116*). The

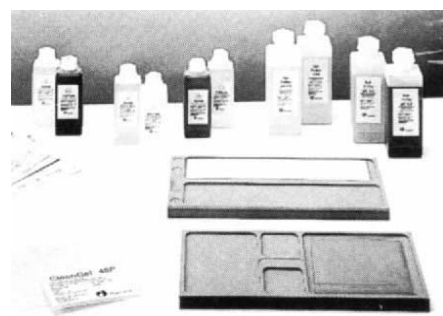


Pulsed-field electrophoresis system.

system electronically generates included angles from 90°–120° in one-degree increments, which enables high-resolution separations of DNA molecules ranging from 1 kb to greater than 6 Mb. Up to three consecutively executing blocks of run conditions can be programmed into the CHEF DR III, each block with run parameters optimal for each segment of the total size range. To simplify programming, the CHEF DR III contains a power supply, switcher and electronic driver in a single power module. In addition, a battery back-up RAM has been installed so that in the event of a power failure the run will automatically resume after the power is restored. Like its predecessors, the CHEF DR III is dynamically regulated to maintain homogeneous electric fields and field angle in the electrophoresis cell, regardless of fluctuations in buffer temperature, pH or conductivity. The system comes complete with the variable-angle power module, electrophoresis cell, variable-speed pump and accessories.

■ Ready-to-use gels

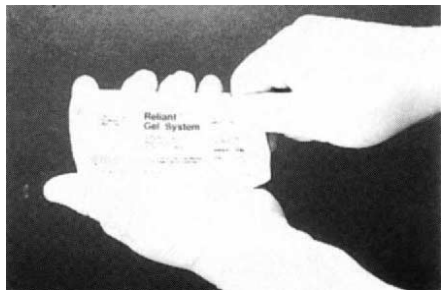
Pharmacia says that its CleanGel **precast gel** is cast, washed and dried under optimal conditions so that it retains no catalysts or toxic nonpolymerized monomer (*Reader Service No. 117*). For added versatility, this type of gel can be re-swollen in the buffer of your choice. CleanGel is supplied to the



Precast CleanGels are ready to run.

user empty so that it can be tailored to specific applications. In addition, CleanGel has built-in sample wells for 16–48 samples and up to 100 µl sample volumes. Pharmacia offers specific CleanGel buffer kits for SDS electrophoresis, native acid electrophoresis in the cathodal and anodal direction, and for basic electrophoresis in the anodal direction. CleanGel is also designed to be fast. Because it is thin (0.5 mm), Pharmacia says that it runs, stains and destains very quickly, using very little buffer solution.

The Reliant gel system from FMC BioProducts consists of ready-to-use **precast agarose minigels**, which are available in a variety of concentrations and buffers (*Reader Service No. 118*). FMC BioProducts says

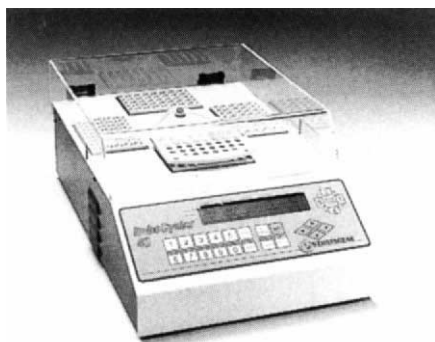


Reliant precast agarose minigels.

that use of its Reliant precast agarose gels will enable the user to be ready to run DNA and PCR samples in less than 5 min.

■ Labware

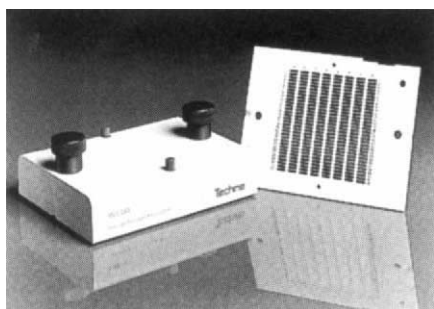
Stratagene says that its RoboCycler 40 **temperature cycler** provides rapid thermal cycling and well-to-well temperature uniformity using a design that robotically moves sample tubes from one temperature block to another (*Reader Service No. 119*). The total cycle time for a typical 30-cycle protocol is reduced by 30–40 min as compared to more conventional thermal cyclers, says Stratagene. Well-to-well temperature uniformity of each of the four blocks is kept to within ± 0.1 °C. The system's three hot blocks can be programmed for a temperature range of 25 °C to 99 °C. The fourth block is thermoelectrically cooled and so can be programmed from 6 °C to 20 °C. Each block contains 40 wells, which can



Stratagene's RoboCycler 40.

accommodate a wide range of 0.5-ml flip-top tubes. Microprocessor control of the RoboCycler 40 system lets the user create up to 99 customized cycling programs, which can be modified during runs or executed unattended overnight. Program information entered by the user is displayed on a large liquid-crystal display, which updates run time and cycle status in real time during execution of the program. The RoboCycler 40 is intended for a wide range of temperature cycling applications including the safe cycling of radioactive samples, ligations and any incubation reaction requiring precise control of both time and temperature.

Based on a semi-dry blotter, the **multi-sample electroeluter**, Model MSE-256, from Techne allows the simultaneous elution of a range of macromolecules from one-dimensional gels, including proteins, nucleic acids and oligosaccharides (*Reader Service No. 120*). The unit has a multiwell matrix that collects purified components from convenient size gels and holds them in



Multisample electroeluter — see Techne.

solution; after elution, samples can be removed either with a single tip or multi-channel pipette for further studies. The MSE-256 is intended for large-scale screening work, in addition to routine protein investigations. The unit uses a normal electrophoresis power supply. Techne says that most proteins between 0 and 120 kDa are recovered in 30 min. Each well of the matrix plate holds 200 µl. By removing the multiwell matrix plate, the unit can be used as a semi-dry blotter. One safety feature is an interlock that is designed to prevent the

cover on the unit from being removed while the power supply is still connected.

Owl Scientific announces the new **ethidium bromide solution and safety dispenser** (*Reader Service No. 121*). The premixed solution is provided in a dose-controlled dispenser bottle to protect the researcher from contact with mutagenic ethidium bromide. A dosage control button dispenses 1 ml of the 200 µg ml⁻¹ aqueous solution. The safety container is also designed to protect the ethidium bromide solution from light and air, providing safe laboratory storage. The ethidium bromide solution and dispenser is available in 25- and 100-ml quantities.

Analtech's Uniscan **video densitometer** for thin-layer chromatography (TLC) is now supplied with an IBM 386DX compatible computer with 4 MB RAM, a 105 MB hard drive, 3.5-inch and 5.25-inch disk drives and a super VGA colour monitor (*Reader Service No. 122*). Additionally, a ×0.5 wide-angle lens and a high-output, white-light box are now included as standard features.



Analtech's Uniscan provides a video view.

Uniscan uses a video camera and IBM-compatible computer to scan TLC plates or electrophoresis gels. Area values from resultant chromatographic peaks are calculated and can be used to create automatically standard curves. Unknown values are then calculated and displayed. The system comes complete with all the software

and hardware (including computer) needed to begin scanning.

■ Services and handbooks

British Bio-technology now offers a range of **custom services** to meet the needs of researchers whose time, resources or experience may be limited (*Reader Service No. 123*). BBT's confirmatory DNA sequencing service provides a sequence printout and autoradiographs of all sequencing gels. Sanger dideoxy sequencing is performed manually to ensure accuracy and facilitate resolution of ambiguous sequences. The company's plasmid manipulation service includes construct building, gene isolation, gene insertion into a vector, addition of antibiotic resistance, linker addition, cassette building, fragment preparation and site-directed mutagenesis. BBT will also undertake partial or complete gene construction. Probes, amplification primers and oligonucleotides for construction of full-length genes can be designed.

Dynal announces the availability of the first edition of its **technical handbook on biomagnetic separation** (*Reader Service No. 124*). This 48-page application manual includes documentation on the Dynabeads biomagnetic separation system as it applies to molecular biology. It features recommended protocols, schematic diagrams, plots, technical tips and references to published papers up to July 1992. The use of Dynabeads, as a magnetic solid phase, coated with oligonucleotides or with streptavidin has found application in the separation, concentration, identification, detection, quantification or purification of nucleic acids (DNA/RNA). □

These notes are compiled by Diane Gershon from information provided by the manufacturers. For more information, fill in the reader service card bound inside the journal. Prices quoted are sometimes nominal and apply only within the country indicated. The polymerase chain reaction is covered by patents owned by Hoffmann-La Roche, Inc.

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